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Membrane binding controls ordered self-assembly of animal septins

1 **Membrane binding controls ordered self-assembly of animal septins**

2

3 Agata Szuba^{1#}, Fouzia Bano^{2§}, François Iv³, Manos Mavrikis^{3*}, Ralf P. Richter^{2*}, Aurelie Bertin^{4,5*}, Gijssje H.
4 Koenderink^{1,6*}

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 ³Institut Fresnel, CNRS, Aix-Marseille Univ, Centrale Marseille, 13013 Marseille, France

12 ⁴Laboratoire Physico Chimie Curie, Institut Curie, PSL Research University, CNRS UMR168, 75005, Paris, France

13 ⁵Sorbonne Université, 75005, Paris

14 ⁶Department of Bionanoscience, Kavli Institute of Nanoscience Delft, Delft University of Technology, Van der
15 Maasweg 9, 2629 HZ Delft, The Netherlands

16

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

19 #Current address: Pollard Institute, School of Electronic & Electrical Engineering, University of Leeds, Leeds, LS2 9JT,
20 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 membrane binding requires electrostatic interactions of septins with anionic lipids**
27 **and promotes the ordered self-assembly of fly septins into filamentous meshworks. Transmission**
28 **electron microscopy reveals that both fly and mammalian septins form arrays of single and paired**
29 **filaments. Atomic force microscopy and quartz crystal microbalance demonstrate that the fly filaments**
30 **form mechanically rigid, 12 to 18 nm thick, double layers of septins. By contrast, C-terminally truncated**
31 **septin mutants form 4 nm thin monolayers, indicating that stacking requires the C-terminal coiled coils**
32 **on DSep2 and Pnut subunits. Our work shows that membrane binding is required for fly septins to form**
33 **ordered arrays of single and paired filaments and provides new insights into the mechanisms by which**
34 **septins may regulate cell surface mechanics.**

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35 Introduction

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

50 It remains unclear whether the cortical septin pool in animal cells associates with the plasma
51 membrane itself or with membrane-bound actin filaments and microtubules [35, 36]. Biochemical
52 reconstitution studies have shown that mammalian septins can bind phosphoinositides [4, 37-40], but
53 septins have also been shown to bind actin filaments [41-43] and various actin-binding proteins including
54 anillin [44] and non-muscle myosin-2 [45]. Electron microscopy of immuno-stained cells revealed
55 localization of cortical septins with cortical actin in tissue culture cells [36, 46], but the high density of the
56 actin cortex in animal cells has made it impossible to determine whether cortical septins directly interact
57 with the plasma membrane. It is even unclear whether cortical septins truly form filaments. Fluorescence
58 microscopy has shown the presence of cortical septin puncta, fibrous strands, or rings [25-27, 29], but has
59 lacked the resolution to resolve the precise nature of these structures. Septin-deficient cells exhibit a
60 markedly reduced cortical rigidity [25-27, 29], but it is unclear whether these effects imply a loss of
61 membrane-bound septin filaments or a loss of septin-mediated actin stabilization. A further complication
62 is that microtubules have also been found to interact with cortical septins [47].

63 To resolve the functions of septins at the animal cell cortex, it is necessary to understand the
64 innate ability of septins to assemble alone and in association with the membrane. Several reconstitution
65 studies have been reported for native and recombinant septins from various animal species, but nearly
66 all of these studies considered septin assembly in bulk solution. Frog (*Xenopus laevis*) septins were found
67 to form paired filaments similar to yeast septins [48], while recombinant nematode (*C. Elegans*) septins
68 [49] and native and recombinant fly (*Drosophila melanogaster*) septins [41, 50, 51] were observed to form
69 bundles. Recombinant mammalian septin hexamers have been shown to form rings and spirals that are
70 reminiscent of structures formed by Shs1-capped yeast septin octamers [52]. To the best of our
71 knowledge, there are only two reports until now of the organization of animal septins on lipid membranes,
72 showing that septins in porcine brain extracts can tubulate giant liposomes and form filamentous
73 meshworks encasing the membrane tubes [38, 40].

74 Here we investigate the role of membrane-binding in septin assembly by reconstituting
75 recombinant animal septins on flat supported lipid bilayers. We focus on septin hexamers composed of
76 Pnut, DSep2, and DSep1 from the model organism *Drosophila*. We studied the septins on model cell
77 membranes composed of phosphatidylcholine (PC) lipids combined with either phosphatidylserine (PS) or

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78 phosphatidylinositol(4,5)-bisphosphate (hereafter referred to as PI(4,5)P₂). PS is the most abundant
79 anionic lipid in the inner leaflet of the plasma membrane [53]. The cell membrane in the fly has been
80 reported to contain 3-10% PS, but the composition varies among tissues and developmental stages [54-
81 56]. PI(4,5)P₂ is an anionic signaling lipid previously shown to interact with yeast and mammalian septins
82 [37, 57, 58] and assumed to be important for septin-membrane interactions in the fly [59, 60]. We show
83 by fluorescence imaging that fly septin hexamers have a high affinity for membranes containing PS and/or
84 PI(4,5)P₂, and form dense membrane-associated meshworks. Nanometer-resolution imaging by electron
85 microscopy and by atomic force microscopy reveals that these meshworks comprise single and paired
86 filaments, which laterally associate into bundles that form tightly packed domains. We propose a
87 molecular model of the septin self-assembly mechanism that is consistent with these new data. Our
88 findings establish that animal septins have the innate ability to form stable filamentous meshworks on
89 lipid membranes, which likely contributes to their ability to regulate cell cortex rigidity and tension.

90

91 Results

92

93 Septins form bundles in solution

94 To understand how membranes influence septin assembly, we begin by analyzing how septins assemble
95 in free solution. We focus on septin hexamers composed of DSep1, DSep2, and Pnut from the model
96 organism *Drosophila*, which have been previously characterized *in vivo* [41, 61-63] and *in vitro* [41, 50,
97 51], and which are highly homologous to their human septin orthologs (DSep1, DSep2 and Pnut are 75%,
98 68% and 65% identical to Sept2, Sept6 and Sept7, respectively; see Methods section). Based on
99 phylogenetic analysis that classifies fly and human septins in distinct subgroups (DSep1/Sept2 in the SEPT2
100 subgroup; DSep2/Sept6 in the SEPT6 subgroup; Pnut/Sept7 in the SEPT7 subgroup [64, 65]), and the order
101 of subunits in mammalian septin complexes [66, 67], fly septin hexamers should have a palindromic
102 arrangement with the Pnut subunits in the center, the DSep1 subunits at the termini, and the DSep2
103 subunits in between (**Figure 1C**). Each subunit carries a flexible carboxy-terminal extension, a part of which
104 is predicted to adopt a coiled-coil arrangement [68]. Using the coiled-coil prediction algorithm COILS (see
105 Methods section), we predict the C-terminus of DSep1 to form a coiled-coil that is 28 residues long, and
106 the C-termini of Pnut and DSep2 to form coiled-coils that are 86 residues long. Our coiled-coil prediction
107 analysis for human septins yields the same predictions, *i.e.*, 28-residue-long coiled-coils for Sept2 and 86-
108 residue-long coiled-coils for each Sept6 and Sept7 (**Figure 1B**). Considering a 1.5 Å rise per residue in an
109 alpha-helix, we estimate the coiled-coil of DSep1/Sept2 to be 4 nm long, and the coiled-coils of
110 DSep2/Sept6 and Pnut/Sept7 to be 13 nm long (**Figure 1C**). Hexamers form 24 nm-long rods, with the
111 globular domains of the septin subunits approximately 4 nm in diameter [41, 69]. The coiled-coil of DSep1
112 could thus extend as much as the G-domain itself, while the coiled-coils of DSep2 and Pnut could even
113 extend as much as 3-fold the size of the G-domain. From the end of the α 6-helix to the start of the
114 predicted coiled-coils, there are stretches of 24, 15 and 15 residues for DSep1, DSep2 and Pnut,
115 respectively (see **Figure 1A**), that are predicted to be unstructured. Given a rise per residue of ~0.4 nm,
116 these lengths translate to contour lengths of 6 to 10 nm. These regions thus likely act as a flexible hinge
117 between the G-domain and the coiled-coil, allowing the coiled-coils to pivot around their anchor points.
118 This prediction is consistent with observations that coiled coils did not show up in X-ray crystal structures
119 or particle-averaged electron microscopy (EM) analysis of yeast and mammalian septin oligomers [9, 69].

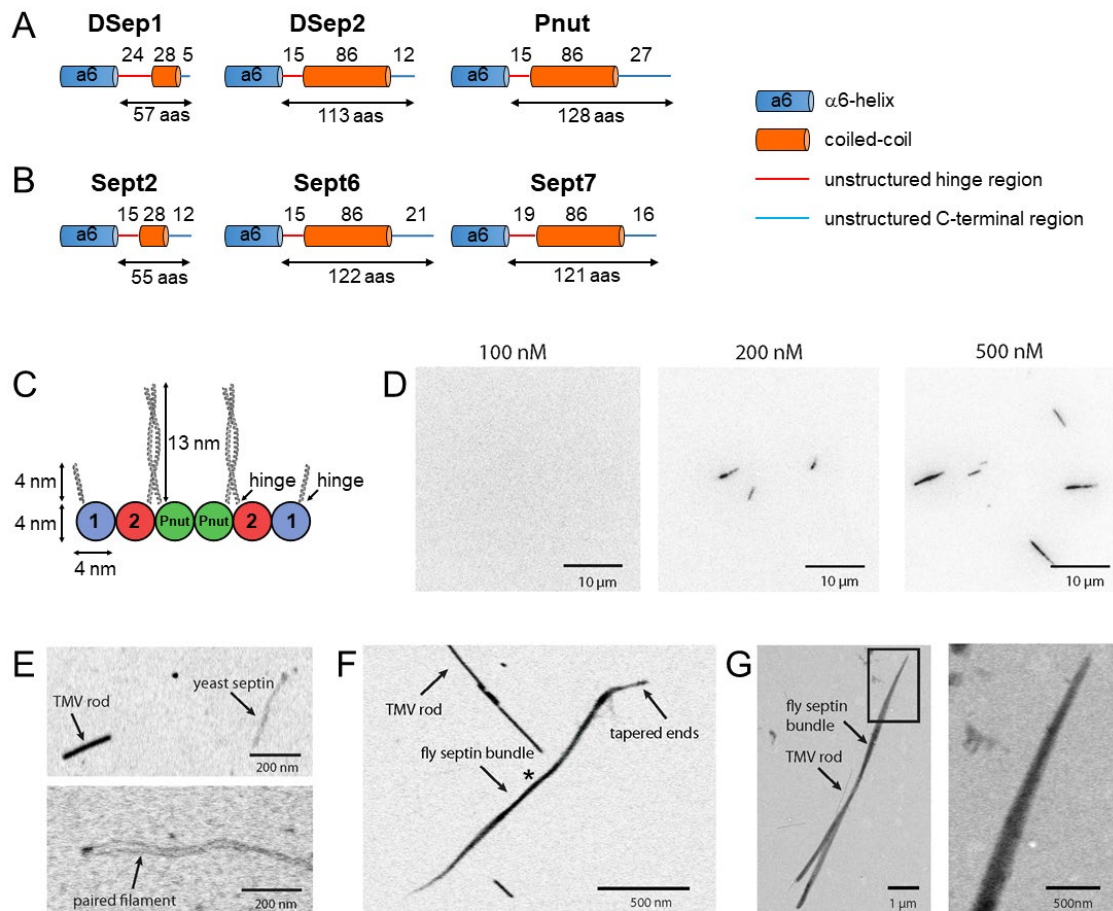
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120 Moreover, EM images of yeast, *Drosophila*, and *C. elegans* septin oligomers showed direct evidence that
121 the coiled coils can sweep out a large volume [9, 41, 49].

122 To test the ability of the recombinant fly septins to polymerize in bulk solution, we performed
123 TIRF imaging of mEGFP-tagged septin hexamers (**Figure 1D**) after rapid dilution from a high salt storage
124 buffer containing 300 mM KCl to a low salt polymerization buffer containing 50 mM KCl. We expect fly
125 septins to form bundles under these conditions [41, 70]. To enable observation of septins in the thin (100
126 nm) evanescent TIRF field, we pushed them down onto a coverslip passivated with a neutral (PC) lipid
127 bilayer with the crowding agent methylcellulose. As shown in **Figure 1D**, septins did not form any
128 structures visible at the resolution of the light microscope until the concentration reached 200 nM. When
129 we increased the concentration further to 500 nM, the septins formed longer bundles. Bundle formation
130 was very rapid, taking less than 2 minutes for completion. The bundles were rather straight and did not
131 exhibit measurable thermal bending undulations, indicating that they must be stiff.

132 To measure the width and mass of the septin bundles, we turned to scanning transmission
133 electron microscopy, which provides quantitative mass measurements based on the dark-field signal from
134 elastically scattered electrons emitted under a high angle [71]. The sample is raster-scanned with a
135 focused electron beam and the intensity in each image pixel is converted to the projected specimen mass
136 by including tobacco mosaic virus (TMV) rods with a well-defined mass-per-length (MPL) of 131 kDa/nm
137 as an internal calibration. To test the accuracy of the mass mapping method, we first imaged budding
138 yeast septins, since these are already known to form paired filaments with a theoretical mass per length
139 of 23.2 kDa/nm [9]. As shown in **Figure 1E**, the yeast septins form thin semiflexible polymers that are
140 weaker in intensity than the TMV rods, consistent with their smaller mass per length. The average mass-
141 per-length (based on 10 septin filaments from 3 images) was ~20 kDa/nm, close to the mass-per-length
142 expected for paired filaments. In rare cases the image clearly showed a double-stranded structure with
143 two filaments running in parallel with a small spacing in the range of 17 nm – 27 nm, consistent with prior
144 transmission EM findings [9] (see lower panel of **Figure 1E**). By contrast, fly septins formed thick bundles
145 that were stronger in intensity than the TMV rods, indicating a larger MPL (**Figure 1F**). Bundles formed at
146 200 nM had tapered ends and a thicker center. The example bundle in Figure 1F has a MPL of around 250
147 kDa/nm in the center (marked by an asterisk) and a corresponding center width of 31 nm. Given a
148 calculated MPL of 12.8 kDa/nm per fly septin hexamer, this number translates to around 20 hexamers per
149 cross-section. Considering that the inter-filament spacing has to be at least 4 nm, a width of 31 nm and
150 20 hexamers per cross-section implies there are at least 3 monolayers. At 500 nM, the septin bundles
151 were thicker (**Figure 1G**) with maximal widths of up to 280 nm and MPL values up to ~5000 kDa/nm (**Figure**
152 **1 – Figure Supplement 2**), corresponding to >400 hexamers per cross-section or a thickness of at least 6
153 monolayers. This type of analysis suggests that septin bundles in solution do not grow as flat sheets but
154 as 3D bundles.

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155
 156 **Figure 1: Fly septin hexamers form bundles in solution.** (A) Structure predictions for the C-terminal
 157 regions of fly septins, starting from the end of the $\alpha 6$ -helix regions. (B) Corresponding predictions for
 158 human septins. (C) Schematic of the fly septin hexamer, showing its expected palindromic arrangement
 159 with long (13 nm) coiled-coil extensions of the Pnut and DSep2 (2) subunits, and shorter (4 nm) extensions
 160 on the DSep1 (1) subunits. The predicted septin coiled-coils are depicted to scale using available crystal-
 161 structures of coiled-coils (see Methods). (D) TIRF images of mEGFP-tagged fly septin bundles formed in
 162 solution and crowded onto net-neutral SLBs composed of PC by methylcellulose, with septin
 163 concentrations as indicated above each image. (E) STEM images of yeast septin filaments. Upper panel:
 164 septin filament and a TMV rod (see arrows). Lower panel: septin filament showing a paired arrangement
 165 of two linear strands. (F) STEM image of fly septin bundle formed at a concentration of 200 nM surrounded
 166 by several TMV rods (one example is pointed out). The bundle center width and MPL (asterisk) are 31 nm
 167 and 250 kDa/nm, respectively. (G) STEM image of a fly septin bundle formed at a concentration of 500
 168 nM, together with three TMV rods (one is indicated by an arrow). Note that TIRF and STEM images are all
 169 contrast-inverted, so septins appear dark against a light background.

170 The following figure supplements are available for figure 1:

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

172 **Figure 1 – Figure supplement 2.** Quantification of the bundle size for septin bundles formed in bulk
 173 solution at 500 nM.

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

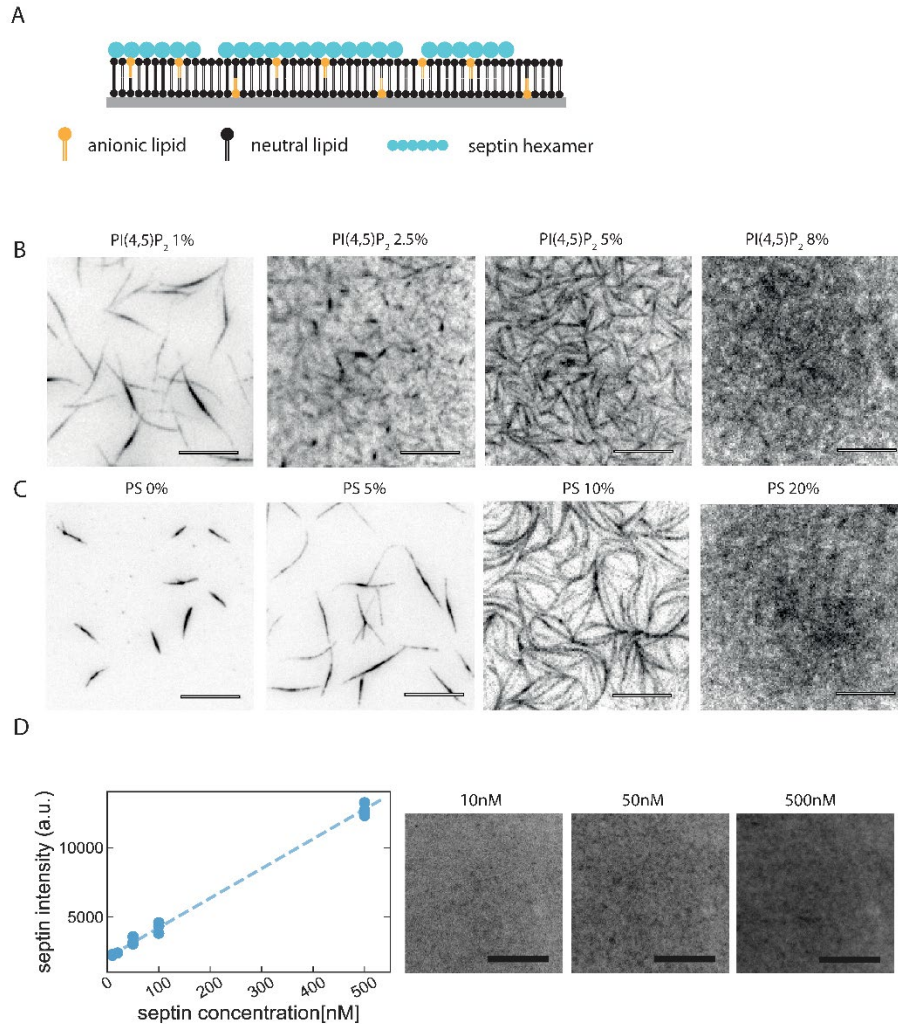
175 To investigate how membrane-binding affects septin polymerization, we deposited the purified fly septin
176 hexamers in polymerization buffer on glass-supported lipid bilayers (SLBs) composed of net-neutral PC
177 lipids doped with anionic lipids (**Figure 2A**). To test whether fly septins bind PI(4,5)P₂, as reported for yeast
178 septins [57, 58], we doped the PC membranes with different mole percentages of PI(4,5)P₂ ranging from
179 1 to 8%. Already at 1% PI(4,5)P₂, septins formed immobile bundles adhering to the membrane (**Figure 2B**).
180 We note that membrane recruitment was observed here in the absence of methylcellulose, showing that
181 septins indeed bind to PI(4,5)P₂ lipids. As we raised the PI(4,5)P₂ content from 1% to 8%, we observed a
182 striking transition from a sparse arrangement of thick bundles to a dense protein layer. At intermediate
183 PI(4,5)P₂ fractions of 2.5% and 5% we could still discern septin bundles, but at 8% PI(4,5)P₂ the protein
184 density was too high to discern any details of the ultrastructure.

185 To test whether fly septins selectively bind the PI(4,5)P₂ head group as reported for yeast septins
186 [57, 58] or simply bind through nonspecific electrostatic interactions, we next replaced PI(4,5)P₂ by PS. As
187 shown in **Figure 2C**, fly septins were also recruited to the PS-containing bilayers and again showed a
188 transition from a sparse distribution of thick bundles at low PS content to a dense layer at high PS content.
189 The adsorbed amount of septins, as quantified from the total fluorescence intensity, linearly increased as
190 the PS content was raised from 5% to 20% (**Figure 2 – figure supplement 1A,B**). The observation that PS
191 has a qualitatively similar effect compared to PI(4,5)P₂ on septin recruitment and assembly on SLBs
192 suggests that the membrane-binding affinity of fly septins is governed by the net surface charge of the
193 membrane rather than by any specific affinity for PI(4,5)P₂. The transition from dilute septin bundles to
194 dense septin films required a larger PS mole fraction (10%) than PI(4,5)P₂ (2.5%), consistent with the larger
195 net charge (-4) of the head group of PI(4,5)P₂ due to the phosphate groups compared to the net charge (-
196 1) on the head group of PS [72-75]. Therefore, our findings suggest that the total negative surface charge
197 of the membrane governs septin adsorption, indicating that the septin-membrane interaction is primarily
198 electrostatic in origin.

199 In all cases, filamentous septin structures were already present as soon as we could start imaging
200 (~3 min after septin injection) and the structures did not change in number or thickness over time (**Figure**
201 **2 – figure supplement 1A**). Apparently, septins bind strongly and rapidly to the membrane. Fluorescence
202 recovery after photobleaching experiments showed that the septins exhibited negligible subunit exchange
203 (**Figure 2 – figure supplement 2A**), indicating that they are indeed stably anchored to the membrane. The
204 membrane underneath the septin film was nevertheless fluid, as indicated by FRAP measurements of
205 rhodamine-labeled PE tracer lipids (**Figure 2 – figure supplement 2B**).

206 To test the dependence of septin adsorption on bulk septin concentration, we incubated bilayers
207 containing 20 mole-% PS with solutions of septin hexamers at concentrations ranging between 10 and 500
208 nM. As shown in **Figure 2D**, we observed uniform septin layers with a texture that could not be resolved
209 at the resolution of the TIRF microscope over this entire concentration range. The septin fluorescence
210 intensity increased roughly linearly with septin concentration. Interestingly, septins already formed
211 membrane-bound layers at 10 nM, *i.e.*, at concentrations far below the onset concentration of 200 nM
212 for bundle formation in bulk solution (**Figure 1B,C**), suggesting that membrane association catalyzes
213 polymerization. However, at the diffraction-limited resolution provided by TIRF microscopy we cannot
214 distinguish whether the membrane-bound layers truly are filamentous. At concentrations above 200 nM,
215 we observed the presence of septin bundles in the solution above the bilayer by confocal imaging,
216 consistent with the observed onset for bundling in the absence of an adhesive membrane (**Figure 1D**).

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217

218 **Figure 2: Glass-supported lipid bilayers containing anionic lipids recruit septins and promote the**
219 **assembly of dense septin meshworks.** (A) Purified fly septin hexamers are deposited on a glass-supported
220 bilayer composed of net-neutral (PC) and anionic (PS or PI(4,5)P₂) lipids (sketch not to scale). (B) TIRF
221 images recorded ~3 min after the deposition of mEGFP-tagged septin hexamers (1 μM) on bilayers doped
222 with PI(4,5)P₂ at mole fractions between 1 and 8% (see legend). (C) TIRF images obtained ~3 min after the
223 deposition of septins (1 μM) on bilayers doped with PS at mole fractions between 5 and 20% (see legend).
224 (D) Septins form dense layers on PC bilayers containing 20% PS whose fluorescence intensity increases
225 linearly with the solution concentration. Note that the imaging settings in panel (D) are different from
226 those in panels B,C, so the intensities cannot be directly compared. The dashed line shows a linear fit to
227 the data; the non-zero intercept reflects background fluorescence. Each data point is collected from 1
228 experiment with 3 different regions of interest in different locations. Note that all images are contrast-
229 inverted, so septins are dark and membrane areas devoid of septins are light. Scale bars: 10 μm.

230

231 The following figure supplements are available for figure 2:

232 **Figure 2 – Figure supplement 1.** Dependence of septin adsorption on the PS content of the SLBs.

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

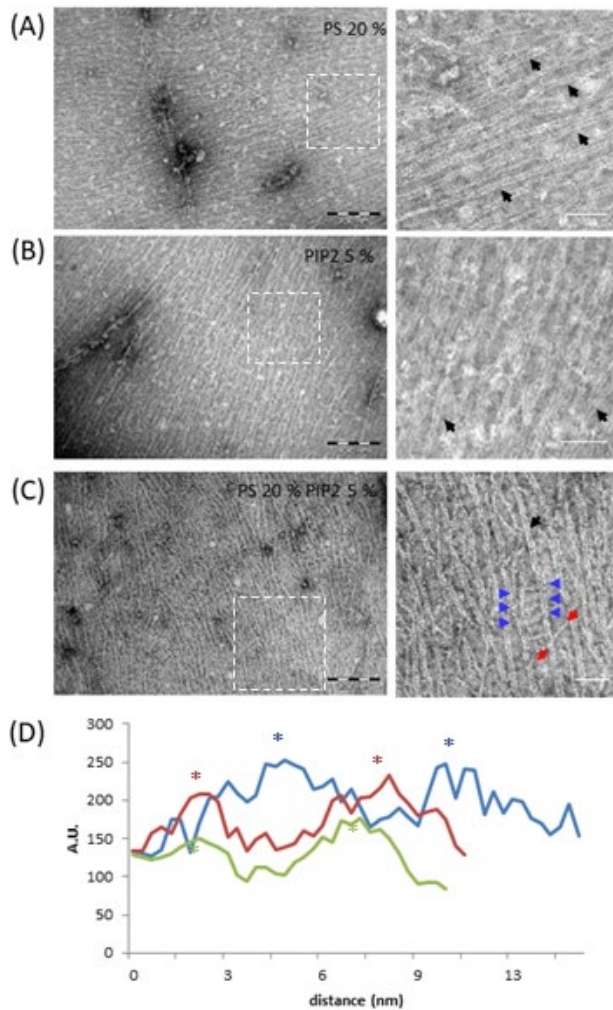
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234 To discern the ultrastructure of membrane-adsorbed septins, we turned to transmission electron
235 microscopy. We incubated septins with lipid monolayers, deposited these on EM grids, and negatively
236 stained the protein with 2% uranyl formate, following a protocol previously used with yeast septins [57].
237 To test the role of membrane composition, we formed lipid monolayers by mixing PC lipids with either 20
238 mol-% PS, 5 mol-% PI(4,5)P₂, or a combination of both that mimics the co-existence of both lipids in the
239 plasma membrane of cells [76, 77]. As shown in **Figure 3A-C**, septins formed densely packed arrays of thin
240 filaments in all three cases, which is qualitatively consistent with the dense meshworks observed by TIRF
241 microscopy. Close inspection of the EM micrographs revealed paired septin filaments (black arrows point
242 out examples). Line profiles revealed an average center-to-center spacing of 5.6 ± 0.7 nm ($N = 57$; see
243 examples in **Figure 3D**). Given a width of 4 nm for fly septin hexamers [41], the spacing between the two
244 filaments forming a pair is only 1.6 nm. Pairing of yeast septin filaments has been reported to occur
245 through antiparallel interactions of coiled coils on opposing filaments [9]. To accommodate the narrow
246 spacing we observe, the longer (~13 nm) coiled coils of the Pnut and DSep2 subunits (**Figure 1C**) of
247 adjacent filaments may arrange anti-parallel and lie flat (i.e. parallel to the lipid plane), or they may
248 arrange parallel and pointing upwards (i.e., away from the lipid plane).

249 On monolayers containing 20% PS plus 5% PI(4,5)P₂, we could observe clear examples of paired
250 filaments that were bridged at intervals of 24 nm by single hexamer rods recognizable by their 24 nm
251 length (**Figure 3C**), forming a filamentous network. These bridges were either perpendicular to the
252 filaments they bridged (blue arrowheads) or under an angle (red arrowheads). We observed comparable
253 arrays of tightly paired filaments connected by orthogonally or diagonally oriented single filaments for
254 mammalian septins on lipid monolayers of the same composition (**Figure 3 – figure supplement 1**). Similar
255 arrays of filaments were also observed for budding yeast septins [52, 57], suggesting that this architectural
256 feature is conserved within eukaryotes. We do note a subtle difference for the mammalian septins as
257 compared to the fly septins. For the fly septins, the bridges between paired filaments are single isolated
258 hexamer rods. Instead, for the mammalian septins, the paired filaments (white arrows in **Figure 3 – figure**
259 **supplement 1**) are intersected with a distinct set of thinner continuous filaments (red arrows), creating a
260 network of interconnected and perpendicular filaments. It is unclear whether the bridging filaments are
261 single or paired; if paired, the filaments could be rotated or twisted to appear thinner.

262 Since the sample preparation for the TEM experiments requires drying and negative staining, we
263 complemented these findings by cryo-EM imaging on large unilamellar vesicles comprised of PC and 6
264 mol-% PI(4,5)P₂ incubated with septin hexamers. As shown in **Figure 4**, the cryoEM images confirm the
265 presence of paired septin filaments on PI(4,5)P₂-containing lipid membranes (examples are traced out by
266 double red lines in **Figure 4A, B**). The average center-to-center distance between the filaments forming a
267 pair was 5.7 ± 0.8 nm ($N = 40$), in excellent agreement with the negative stain images of septin filaments
268 on lipid monolayers (see **Figure 4 – figure supplement 1**). In addition, we occasionally observed single
269 filaments or hexamers (examples are traced out by single red lines in **Figure 4B**) including some that
270 appeared to interconnect filaments at an orthogonal or oblique angle (blue lines in **Figure 4B**). The density
271 of filaments varied among vesicles (see **Figure 4** and **Figure 4 – figure supplement 2**), which might be due
272 to some variability in the proportion of charged lipids incorporated within the vesicles. Besides, as
273 demonstrated previously [78, 79], the curvature of the vesicles affects the septin-vesicle interaction and
274 most likely introduces additional variability in the density of filaments interacting with vesicles. Vesicles
275 decorated with septin filaments typically appeared to have a faceted contour, whereas vesicles alone
276

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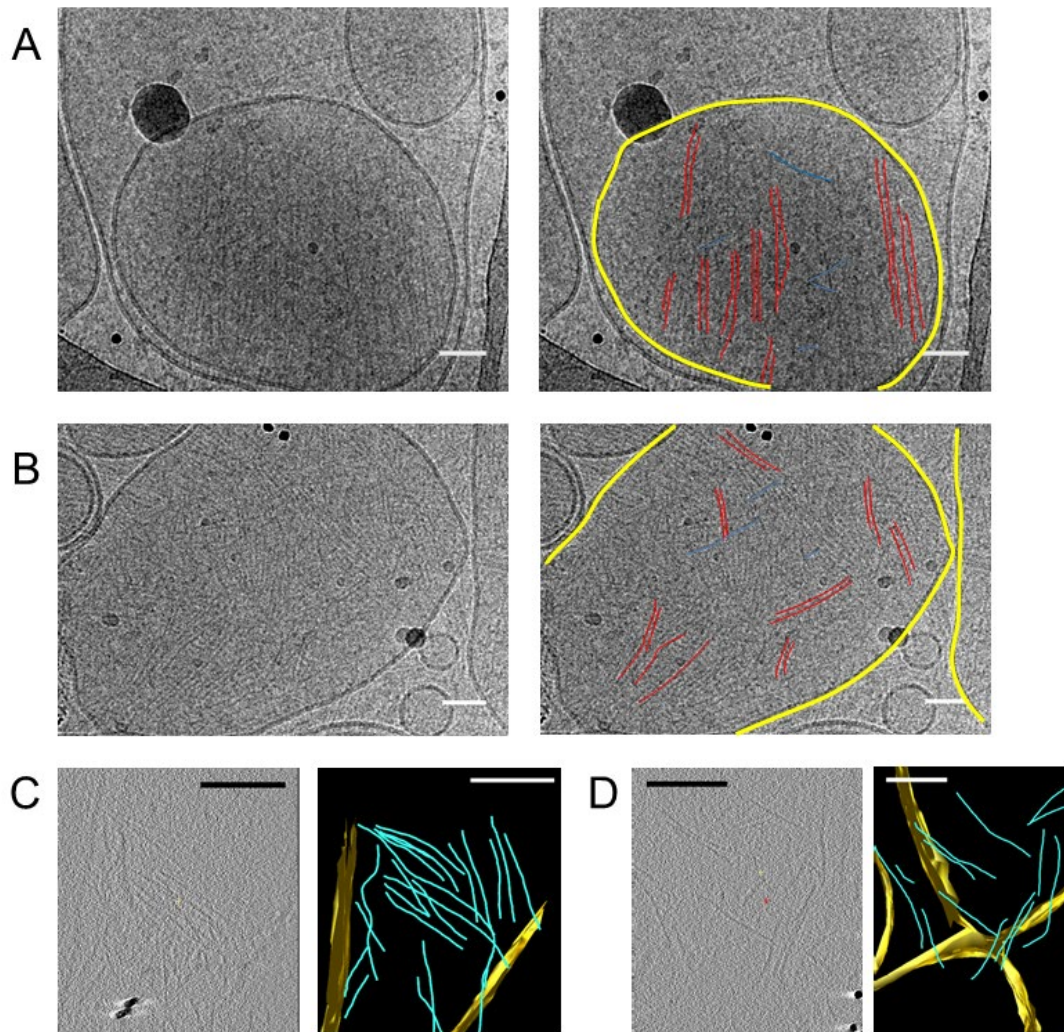


277
278 **Figure 3. Septin hexamers form dense arrays of tightly paired filaments on anionic lipid monolayers.**
279 Electron micrographs of negatively stained fly septin hexamers (65 nM) after overnight incubation with
280 lipid monolayers composed of PC combined with anionic lipids: (A) 20% PS, (B) 5% PI(4,5)P₂, and (C)
281 20% PS plus 5% PI(4,5)P₂. Images on the right show zoom-ins of the regions in the white-dashed boxes on the
282 left. Black arrows point out examples of paired filaments recognizable by two linear filaments running in
283 parallel. Blue arrows indicate examples of orthogonal hexamers, recognizable by their ~24 nm length,
284 between adjacent paired filaments. The two red arrows point to the two ends of a longer (43 nm) cross-
285 bridging filament that bridges two adjacent paired filaments under an oblique angle. Scale bars: 250 nm
286 (left) and 50 nm (right). (D) Three examples of intensity profiles across paired filaments on monolayers
287 containing 20% PS and 5% PI(4,5)P₂PS, showing an interfilament spacing (between stars in matching
288 colours) of approximately 6 nm.

289
290 The following figure supplement is available for figure 3:

291 **Figure 3 – Figure supplement 1.** EM images of mammalian septin hexamers on lipid monolayers.

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292
293 **Figure 4. Septin hexamers form single and paired filaments on large unilamellar lipid vesicles.** CryoEM
294 images of fly septin hexamers (300 nM) after a 30 minute incubation with PC vesicles containing 6%
295 PI(4,5)P₂. (A) Example image (left) with paired septin filaments traced out in red, connecting orthogonal
296 filaments in blue and the vesicle membrane highlighted in yellow (right). (B) Another example image (left),
297 with mostly paired and occasionally single filaments traced out in red, connecting orthogonal filaments in
298 blue and membranes in yellow (right). Black dots are gold nanoparticles that were included as fiducial
299 markers for tomography. The black curved lines in panels A, B come from the carbon lacey substrate. (C)
300 Slice from 3D reconstructed cryo-tomogram (left) with segmented data (right). (D) Another example
301 tomogram (left) with segmented data (right). Reconstructions show membrane in yellow and septin
302 filaments in blue. Note that the bilayer perpendicular to the electron beam is poorly defined because of
303 the missing wedge. Scale bars are 50 nm in A, B and 850 nm in C, D.

304

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

306 **Figure 4 – Figure supplement 1.** Additional cryoEM data.

307 **Figure 4 – Figure supplement 2.** Measurements of interfilament spacing for paired fly septin filaments.

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

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309 usually retained their spherical shape in cryoEM. Note that the bilayer perpendicular to the electron beam
310 is poorly defined because of the missing wedge. The deformed membranes provide a first indication that
311 septins were bound to the membrane and able to flatten it. Some septin filaments clearly protrude a bit
312 beyond the vesicle they adhere to, suggesting they were pre-polymerized in solution and attached to one
313 side of the vesicle without fully wrapping around it. Cryo-electron tomography gave further clear evidence
314 that septin filaments are indeed membrane-bound (see **Figure 4C,D** and **Figure 4 - Movie 1**).

315

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

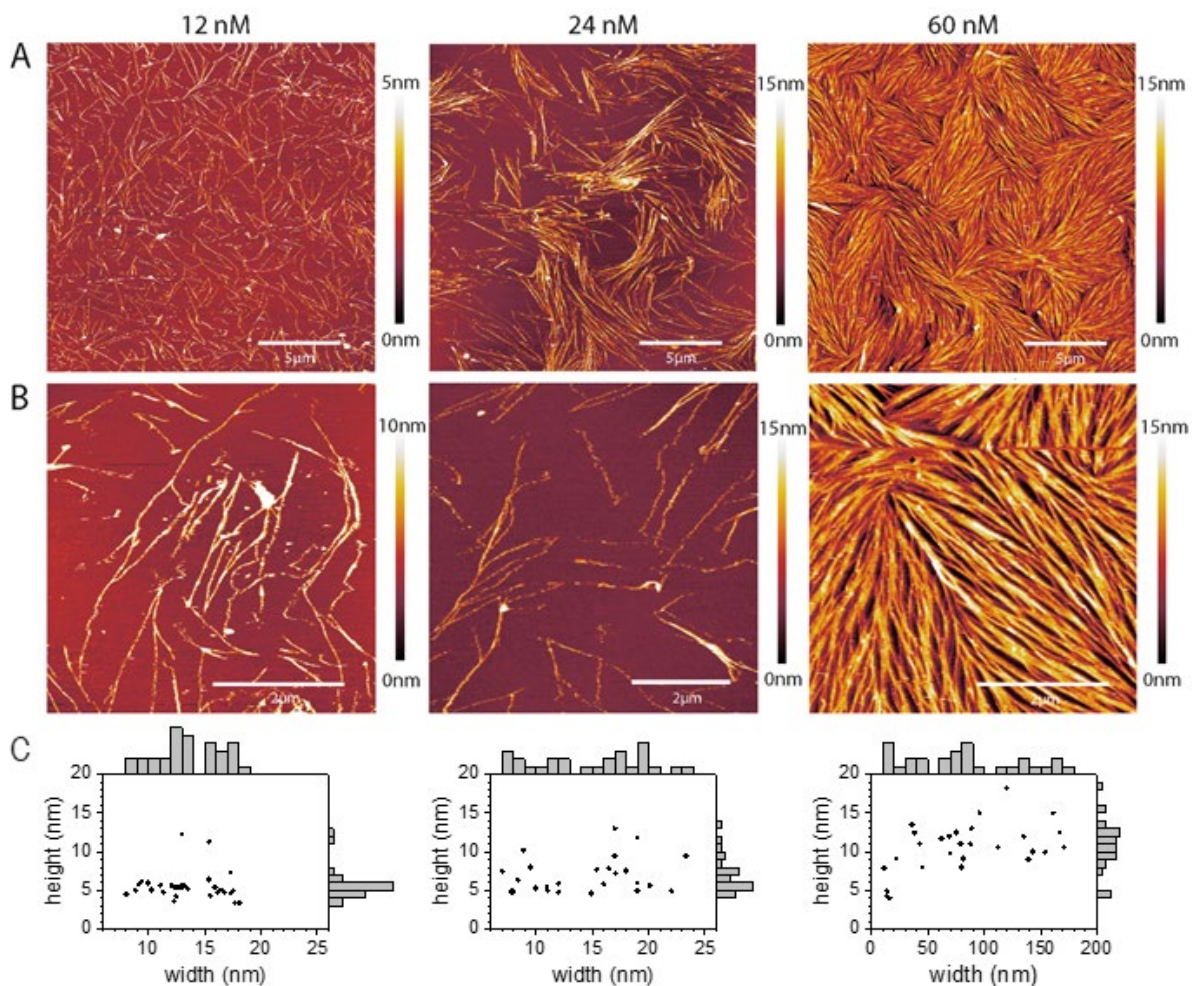
317 We further complemented EM imaging with AFM experiments, which allowed us to image septins on flat
318 lipid bilayers under hydrated conditions resembling the conditions in the TIRF experiments. We performed
319 these experiments on silica-supported lipid bilayers containing 20% PS, which have already been
320 extensively characterized in their quality by AFM and other biophysical techniques [80]. In particular, the
321 even inter-leaflet distribution of PS lipids in SUVs is approximately preserved upon SLB formation on silica
322 (and glass) supports, whereas this is not the case on mica [81], another commonly used support for AFM.
323 We first tested the dependence of septin assembly on septin concentration by imaging septins at
324 concentrations of 12, 24 and 60 nM (**Figure 5A-B**). The samples were fixed with 1% glutaraldehyde (GTA)
325 to prevent septin disruption by the AFM tip.

326 At 12 nM, septins formed threads of typically several micrometers in length that were sparsely
327 and randomly distributed on the bilayer surface (**Figure 5A-B**, left column). These were mostly isolated
328 but could also be seen to meet, mostly at a shallow angle, and merge. Their apparent height was mostly
329 uniform at 5.1 ± 0.9 nm ($N = 29$), with a few notable exceptions around 12 nm ($N = 2$). The apparent width
330 showed two relatively broad but distinct populations: most isolated threads had a width of 11.5 ± 1.6 nm
331 ($N = 19$), whereas all merged threads and a few isolated ones were wider (16.6 ± 0.9 nm; $N = 12$) (**Figure**
332 **5C**, left column, and **Figure 5 – figure supplement 1**). These values are consistent with a mixture of mostly
333 single and paired filaments. This can be appreciated if one considers that the minimal filament height is
334 defined by the extension of the globular domain (4 nm) plus some additional contribution by the coiled
335 coils which, owing to the flexible hinge, may point in various directions and additionally become flattened
336 by the force exerted by the AFM tip. Moreover, the flexing of the coiled coils and tip convolution effects
337 (see Methods for details) can explain the relatively broad width distributions, and why the apparent mean
338 widths exceeded the widths of single and paired filaments as seen by EM by about 8 nm on average.

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

344 At 60 nM, the septins formed threads that densely covered the bilayer surface (**Figure 5A-B**, right column)
345 in closely apposed patches of aligned filaments, resembling nematic domains observed for other 2D arrays
346 of densely packed semiflexible biopolymers [82]. Salient features of the patches were that the constituent
347 threads varied in height and width, and that they formed a network that is characterized by threads
348 frequently joining and disjoining at a fairly shallow angle of approximately 15 degrees. The smallest thread
349 heights and widths observed were comparable to those observed at 12 nM, indicating isolated single or
350 paired filaments were still present. Other threads appeared much wider (up to a few 100 nm; **Figure 5C**,
351 right column). This suggests that many (up to several tens of) single filaments may

Membrane binding controls ordered self-assembly of animal septins



352
353 **Figure 5. Septins form filaments and ordered arrays on lipid bilayers composed of 80% PC and 20% PS.**
354 (A-B) AFM topographic images of membrane-bound fly septin structures formed at solution
355 concentrations of 12 nM (left), 24 nM (middle) and 60 nM (right) and observed at a scan size of (A) 20 ×
356 20 μm². (B) Same samples, imaged at a scan size of 5 × 5 μm² (left and right) and of 6.7 × 6.7 μm² (middle).
357 Color bars on the right show the height scale. The samples were fixed with glutaraldehyde (GTA). (C)
358 Scatter plots with marginal histograms of thread widths and heights determined from the corresponding
359 images in (B). $N = 31$ (left), 26 (middle) and 27 (right) measurements were taken, respectively, across
360 representative sets of well resolved threads.

361

362 The following figure supplements are available for figure 5:

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

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

365 **Figure 5 – Figure supplement 3.** Experiment testing the mechanical stability of the septin structures.

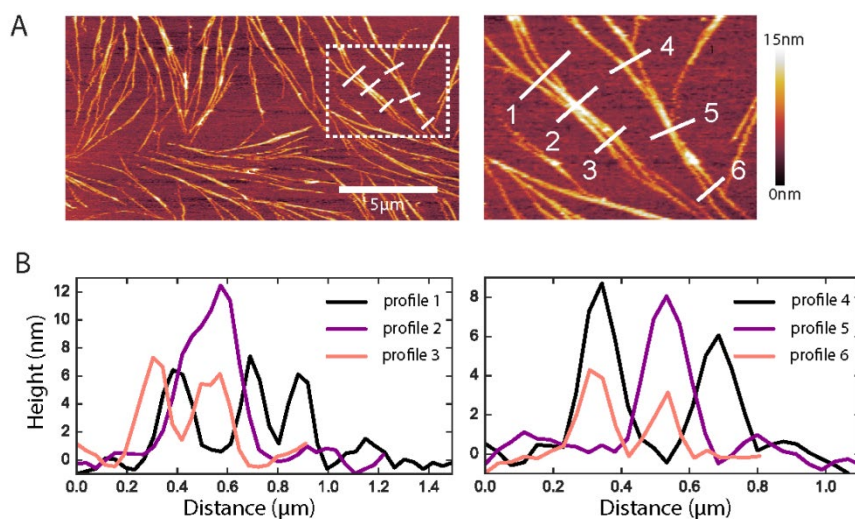
366 **Figure 5 – Figure supplement 4.** Experiment testing the adhesion of septin filaments to the membrane.

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367 closely align on the lipid membrane, although the lateral resolution was insufficient to reveal the
368 individual filaments and their spacing. The wider threads also had an elevated height, mostly between 8
369 and 13 nm and occasionally up to 18 nm (**Figure 5C**, right column), suggesting that septins also stacked on
370 top of each other.

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

383 Since AFM imaging involves mechanical scanning across the surface, it allowed us to qualitatively
384 test how firmly the septins are attached to the lipid bilayer by performing multiple consecutive scans. In
385 sparsely covered bilayer regions, wider bundles generally remained stable whereas narrower bundles or
386 isolated filaments (both GTA-fixed and unfixed) sometimes appeared ragged, suggesting that the AFM tip
387 moved them along the membrane (**Figure 5 – figure supplement 3A,B**). Densely covered regions formed
388 at 60 nM septins were completely unchanged after three consecutive 10-minute scans (**Figure 5 – figure
389 supplement 3C**). These observations suggest that lateral associations among septin filaments within



390
391 **Figure 6. Native ultrastructure of septin assemblies on lipid bilayers composed of 80% PC and 20% PS.**
392 (A) AFM image of non-fixed septins at 60 nM, showing a bilayer region that happens to be sparsely covered
393 with septin filaments. Scale bar for the left panel: 5 μm ; right panel shows zoom of the dashed rectangle
394 in the left panel; color bar on the right shows the height scale. (B) Height profiles were determined along
395 the numbered white lines in the images in A.

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396 bundles or dense arrays cause mechanical stabilization. Some image sequences clearly showed that entire
397 bundles can be laterally displaced along the membrane owing to lateral forces exerted by the AFM tip.
398 Interestingly, these events did not destroy the bundles but resulted in permanent local kinks, suggesting
399 the septin filaments are intrinsically stiff and ductile but the link to the membrane is fluid (**Figure 5 – figure**
400 **supplement 4**).

401

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

403 The AFM data suggest that fly septins form organized layers of stiff filaments on PS-containing bilayers,
404 with a limited thickness. The maximum thickness of the septin films at 60 nM is approximately 18 nm,
405 according to the height profiles. However, we note that this value may underestimate the actual
406 geometrical height because the tip can potentially indent or otherwise disturb the septin layer. To
407 independently measure the film thickness, and to gain insight into the kinetics of septin film formation,
408 we therefore turned to quartz crystal microbalance with dissipation monitoring (QCM-D), an acoustic
409 technique that can monitor the binding of biomolecules and variations in the mechanical properties of
410 interfacial films in real time [83]. We first formed an SLB on the silica-coated QCM-D sensor by perfusing
411 the channel with a vesicle (SUV) suspension and next perfused with a solution of septin hexamers. To
412 investigate the kinetics of SLB formation and septin binding, we monitored the resulting shifts in the
413 resonance frequency Δf , which is proportional to the areal mass density of adsorbed biomolecules plus
414 hydrodynamically coupled solvent, and in the dissipation ΔD , an indicator of the mechanical properties of
415 the adsorbed layer.

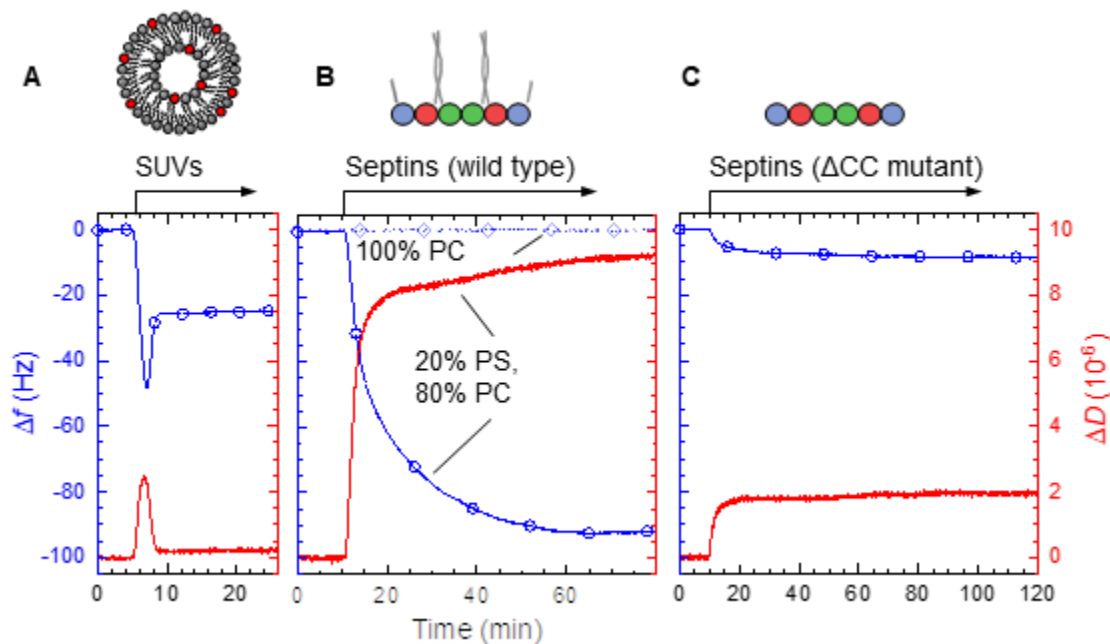
416 Typical QCM-D data are presented in **Figure 7**. The SUVs are composed of 80% PC and 20% PS, as
417 in the AFM experiments. SUV perfusion on a plain silica surface (**Figure 7A**) caused immediate changes in
418 Δf and ΔD in a two-stage process that is characteristic for the initial adsorption of intact SUVs to the sensor
419 surface followed by SUV rupture, spreading and coalescence [84]. The final shifts of $\Delta f = -25 \pm 1$ Hz and
420 $\Delta D < 0.2 \times 10^{-6}$ ($N = 6$) are typical for confluent SLBs composed of PC and PS of a high quality, *i.e.*, with a
421 negligible quantity of residual intact vesicles [80]. Subsequent rinsing with vesicle buffer did not result in
422 appreciable changes in Δf and ΔD confirming the SLB was stable. Septin perfusion at 60 nM on such a SLB
423 (**Figure 7B**) produced an immediate decrease of Δf and a concurrent increase of ΔD , indicating that septins
424 adsorbed. Binding reached a plateau after about 50 minutes, and subsequent perfusion with buffer
425 caused no appreciable change in Δf , suggesting that septins stably adsorb to the membrane. In contrast,
426 we did not observe any adhesion of septins to pure PC membranes (**Figure 7B**; dotted blue line with
427 diamonds). We conclude that septins strongly bind to lipid bilayers containing 20% PS, and that the
428 presence of negatively charged lipids is required for membrane binding, consistent with the TIRF
429 observations.

430 The total frequency shift for septin binding was $\Delta f = 93 \pm 4$ Hz ($N = 4$). Using the Sauerbrey equation
431 (see Eq. [1]), we estimate a film thickness of 15 nm from the frequency shift. This value is consistent with
432 the upper end of bundle heights measured by AFM (**Figure 5C**, right column), which we had attributed to
433 stacking into more than one layer. Moreover, the QCM-D frequency trace also shows that a plateau is
434 reached upon prolonged perfusion with septins. This indicates the presence of a mechanism that limits
435 septin binding and film thickness growth.

436 Finally, QCM-D data are consistent with the septin assembly having soft molecular linkers within
437 the structure. To a first approximation, the $\Delta D / -\Delta f$ ratio is a measure of elastic compliance (*i.e.*, softness)
438 [83], and for globular proteins the major source of compliance typically are flexible hinges linking the

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439 proteins to the surface or inter-connecting protein domains [83, 85]. Membrane-bound septins attained
440 dissipation over frequency shift ($\Delta D/-\Delta f$) ratios between $(0.20 \pm 0.01) \times 10^{-6}/\text{Hz}$ at low coverage ($\Delta f = -15$
441 ± 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 1**).
442 For comparison, monolayers of streptavidin attain $\Delta D/-\Delta f$ ratios of $<0.01 \times 10^{-6}/\text{Hz}$ when physisorbed on
443 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 *via*
444 biotins on a short, flexible linker to a supported lipid bilayer [85]. The higher $\Delta D/-\Delta f$ ratios for septins are
445 consistent with a high degree of flexibility in the linkage between the coiled coils and the globular domains
446 of septin hexamers. Indeed, $\Delta D/-\Delta f$ ratios close to $0.1 \times 10^{-6}/\text{Hz}$ have been reported for monolayers of
447 neutravidin with short (*i.e.* several ten basepairs long) double-stranded DNA strands grafted to it [86], and
448 for monolayers of streptavidin with linear oligosaccharide chains grafted to it [87]. Possibly the
449 compliance of the coiled coils themselves, and/or the linkage between septins and the membrane, also
450 contribute to the elevated $\Delta D/-\Delta f$ ratio relative to fully globular proteins.



451
452 **Figure 7. QCM-D measurements of fly septin hexamer adsorption on supported lipid bilayers (SLBs)**
453 **containing anionic lipids show that septins form thin, rigid films.** Shown are frequency shifts (Δf – blue
454 lines with symbols) and dissipation shifts (ΔD – red solid lines). Arrows on top of the graphs indicate start
455 and duration of sample perfusion; during remaining times, plain buffer (A – vesicle buffer; B and C – septin
456 polymerization buffer) was flown over the sensor surface. (A) Small unilamellar vesicles (SUVs at 50
457 $\mu\text{g}/\text{mL}$; 20% DOPS, 80% DOPC) were exposed to a plain silica surface to form a SLB. (B-C) Hexamers of wild
458 type septin (B) and the ΔCC septin truncation mutant (C) (60 nM each), were exposed to 80% PC/ 20% PS
459 SLBs (solid line with circle symbols). (B) also shows control frequency data for exposure of wild type septin
460 (100 nM) to pure DOPC SLBs (dotted line with diamond symbols).

461
462 The following figure supplement is available for figure 7:

463 **Figure 7 – Figure supplement 1.** Coverage-dependent $\Delta D/-\Delta f$ ratios for fly septins.

464

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

466 The data described above collectively demonstrate that fly septins form filaments that interact with each
467 other both in the plane of the membrane, and out-of-plane. Which septin domains mediate these
468 interactions? One candidate are the C-terminal coiled coils. In prior work on yeast septins, coiled coils
469 have already been proposed to be involved in septin filament pairing and, on membranes, in the formation
470 of perpendicular octamer cross-bridges between two paired filaments [9, 57]. A second candidate are the
471 G-domains, which have been proposed to be involved in lateral interactions among yeast septin filaments
472 on membranes [57].

473 In order to test the arrangement of septins in the presence of G-domain interactions alone, we
474 generated coiled-coil truncated fly septin hexamers (Δ CC mutant). Transmission EM confirmed that fly
475 septins with their C-termini truncated from all subunits form stable hexamers (**Figure 1 – figure**
476 **supplement 1B**), similarly to C-terminally truncated human septin hexamers [69]. TEM imaging showed
477 that in solution, the Δ CC mutant only formed short rods with lengths of ~24-60 nm, corresponding to
478 monomers, dimers or trimers of hexamers, in the low salt polymerization buffer (**Figure 8A,B**). However,
479 C-terminally truncated fly septin hexamers on lipid monolayers formed dense arrays of aligned filaments
480 (**Figure 8C-F**). Thus, membrane-binding promotes filament formation, mirroring earlier observations for
481 yeast septin octamers with C-terminally truncated Cdc11 subunits [57]. In places we can see three
482 filaments side by side instead of two (indicated by triple blue lines in **Figure 8D**). The mutant filaments
483 were even more closely spaced than wild type septin filaments, with a center-to-center distance of around
484 4.5 nm (**Figure 8G**), consistent with direct contact between the globular domains deprived of the CC
485 domain of adjacent filaments due to crowding and perhaps specific G-domain interactions.

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

495

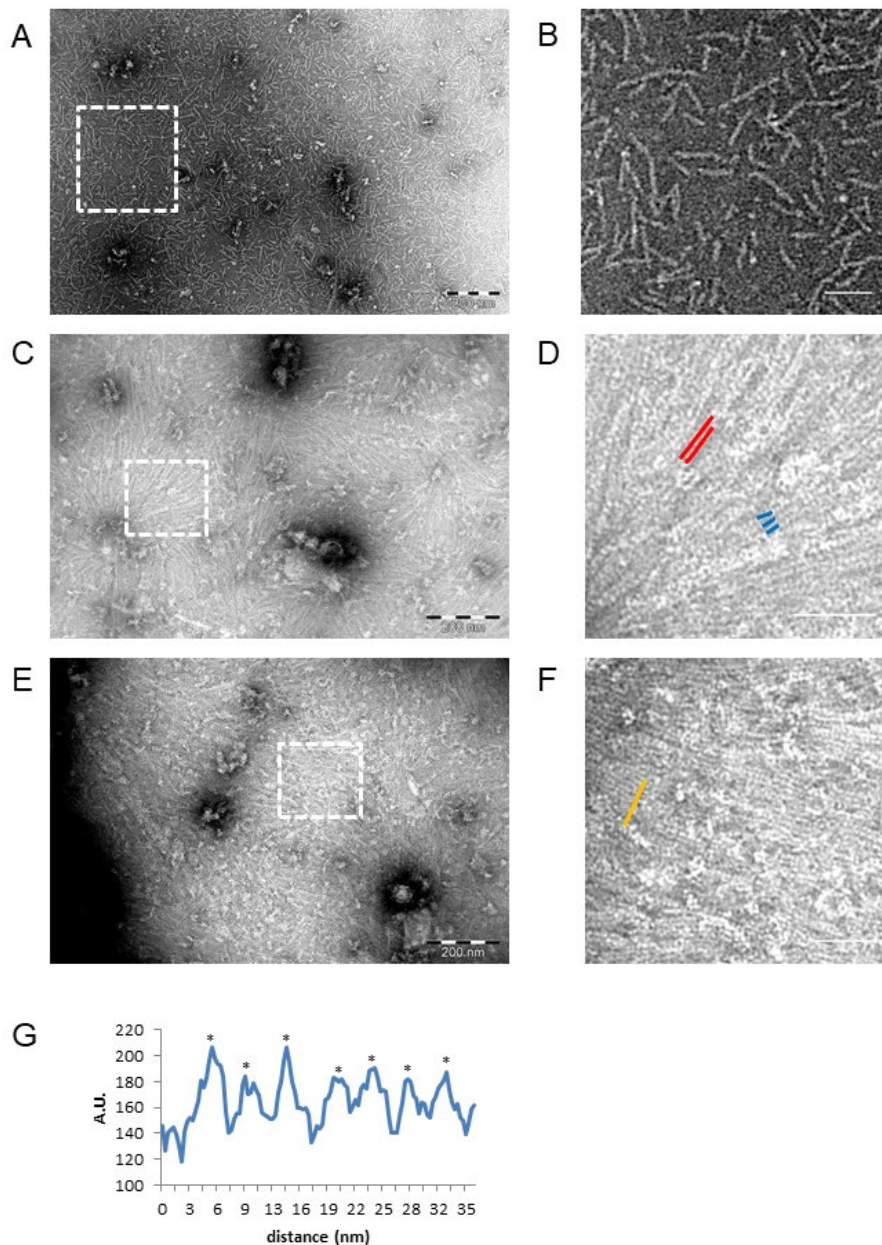
496 **Discussion**

497

498 **Species-dependence of septin-membrane interactions**

499 We investigated the influence of membrane-binding on septin assembly by reconstituting recombinant
500 animal septins on supported lipid bilayers and imaging septin assembly with several complementary
501 techniques. Fluorescence imaging revealed that fly septin hexamers have a high affinity for negatively
502 charged lipid bilayers, which competes with the septin-septin lateral interactions that prevail in bulk
503 solution, and form dense membrane-associated meshworks. Electron microscopy (EM) imaging revealed
504 that these meshworks are predominantly comprised of paired filaments and meshworks thereof. A similar
505 organization was observed for mammalian septins, consistent with earlier findings for septin-containing
506 porcine brain extracts [38, 40]. Finally, atomic force microscopy (AFM) of septins on lipid bilayers showed
507

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508
509 **Figure 8. Negative stain EM images of C-terminally truncated fly Δ CC-septins.** (A) Δ CC-septin hexamers
510 in septin polymerization buffer. (B) Zoom-in of the boxed region in (A) shows that Δ CC-septin hexamers
511 predominantly form monomers and short oligomers of hexamers with a length of ~ 24 -60 nm. (C) Δ CC-
512 septin hexamers (210 nM) on a negatively charged lipid monolayer composed of 80% DOPC, 10% PS and
513 10% PIP(4,5)P₂. (D) Zoom-in of the boxed region in (C) shows sheets of closely spaced, long filaments (red
514 lines) and occasionally three filaments lined up side by side (trimer highlighted in blue). (E) Another
515 example of the Δ CC-septin hexamers (210 nM) on a lipid monolayer composed of 80% PC, 10% PS and
516 10% PIP(4,5)P₂. (F) Zoom-in of the boxed region in (E) again shows sheets of closely spaced, long, unpaired
517 filaments. (G) Line scan (from area highlighted in panel F by an orange line) reveals a center-to-center
518 (asterisks) spacing of ~ 4.5 nm. Scale bars are 200 nm (A,C,E) and 50 nm (B,D,F).

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519 that septin filaments laterally associate into bundles but also stack on top of each other. AFM imaging,
520 fluorescence recovery after photobleaching and QCM-D experiments indicate that the septins are
521 immobile and firmly attached to the membrane. By contrast, C-terminally truncated septin complexes
522 form monolayers and the filaments do not form pairs and meshworks, showing that the coiled coils of
523 septins are crucial for mediating septin-septin interactions.

524 Prior *in vitro* studies of septin-membrane binding focused mainly on mitotic, Cdc11-capped
525 budding yeast septin octamers [57, 88]. We find several striking similarities between the membrane
526 interactions of animal (fly and mammalian) septin hexamers and Cdc11-capped yeast septin octamers.
527 First, membrane binding in both cases promotes septin polymerization. For fly septin hexamers, the
528 threshold for polymerization is lowered more than 10-fold, from 200 nM in solution to less than 12 nM
529 on negatively charged membranes (in the presence of 20% PS). For yeast septins, a similar enhancing
530 effect was observed on membranes containing PI or PI(4,5)P₂ [57, 88]. The polymerization-enhancing
531 effect of membrane binding is likely due to the increase of the effective septin concentration caused by
532 2D confinement. In addition, membrane binding may orient septins in a manner that promotes their
533 polymerization, as seen for other proteins such as annexin A5 [89]. A second similarity between the
534 behavior of animal and yeast septins on membranes is that they both form paired filaments visible by
535 transmission electron microscopy, with a narrow spacing of less than 2 nm, much narrower than the ~10
536 nm spacing observed for yeast septin filaments formed in solution [9]. A third similarity is that paired
537 filaments of both animal and yeast septins are interconnected by bridges visible by electron microscopy,
538 which appear to be formed by short and thin (single) septin filaments having a length and axial spacing
539 that correspond to the length of a single protomer [57]. Similar arrays have been observed by electron
540 microscopy in yeast cells [90], but not yet in animal cells. Note that yeast septins were studied only by
541 transmission electron microscopy, which provides a 2D-projection. It is unknown whether yeast septins
542 form multilayers on membranes, as we see for fly septins by AFM and QCM-D.

543 Our work also reveals two striking differences between animal and yeast septins. The first
544 difference is their polymerization in solution. Fly septins form thick and rigid bundles with tapered ends
545 in solution, suggesting that the septins have strong lateral interactions that promote bundling. On
546 membranes, however, we observed by TIRF microscopy a gradual transition from dilute arrays of thick
547 bundles to dense arrays of paired filaments with increasing net surface charge. This observation suggests
548 a competition of septin-membrane interactions with lateral septin-septin interactions, which suppresses
549 the formation of thick bundles on membranes. By contrast, yeast septin octamers tend to form paired
550 filaments in solution [9, 57], although they do form bundles under certain conditions [91]. Thus, in solution
551 fly septins have a stronger propensity for bundling than yeast septins, but on membranes the two septins
552 behave similarly. The second striking difference between yeast and animal septins revealed here is their
553 membrane binding selectivity. We find that fly septins form similar structures on membranes containing
554 PS or PI(4,5)P₂, and that the main determinant of fly septin binding and filament organization in both cases
555 is the net surface charge of the membrane. By contrast, Cdc11-capped yeast septin octamers were shown
556 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, 39, 57, 58,
557 79, 88, 92, 93]. The origin of the differences in lipid specificity remains unknown. It is thought that septins
558 interact with negatively charged phospholipids *via* a polybasic region close to the N-terminus that is
559 composed of a sequence of 1 to 7 basic amino acids [37, 92]. This stretch is very similar to polybasic
560 sequences found in gelsolin, profilin, G-protein-coupled receptor kinases and ion channels [37], which are

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561 all reported to interact with PI(4,5)P₂. In the future perhaps molecular dynamics simulations can identify
562 the determinants of lipid selectivity [94].

563

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

565 What do the imaging experiments collectively teach us about the assembly mechanism of fly septin
566 hexamers on lipid membranes? A firm conclusion is difficult because there are still a number of important
567 unknowns. It is still under debate where the membrane-binding region is located [79, 95, 96]. We observe
568 that fly septin hexamers are recruited to the membrane by electrostatic interactions with anionic lipids
569 (PS and/or PI(4,5)P₂). It is generally believed that the N-terminal polybasic domains of septins interact
570 with the membrane, which would leave the coiled coils free to rotate in the half-space above the
571 membrane, although a recent study on yeast septin octamers suggested that membrane binding is aided
572 by an amphipathic helix motif identified on the C-terminal side of the coiled-coil of Cdc12 [79]. In our case,
573 however, we observe that C-terminally truncated septins still bind membranes and our data on full-length
574 septins are consistent with the coiled coils being available for interactions with coiled coils on neighboring
575 septins. Since the coiled coils are connected to the body of the septin complex *via* unstructured regions
576 that can act as hinges, we assume that the coiled coils can rotate in the entire half-plane above the
577 membrane, so they are available for both in-plane and out-of-plane interactions with coiled coils on
578 neighboring septin subunits/filaments. The nature of these interactions is still unknown (*i.e.*, assembly of
579 two vs. four coiled coils, and parallel vs. antiparallel assembly). Nevertheless, our imaging and QCM-D
580 experiments do allow us to present a speculative model of membrane-templated septin self-assembly,
581 which is illustrated in **Figure 9**.

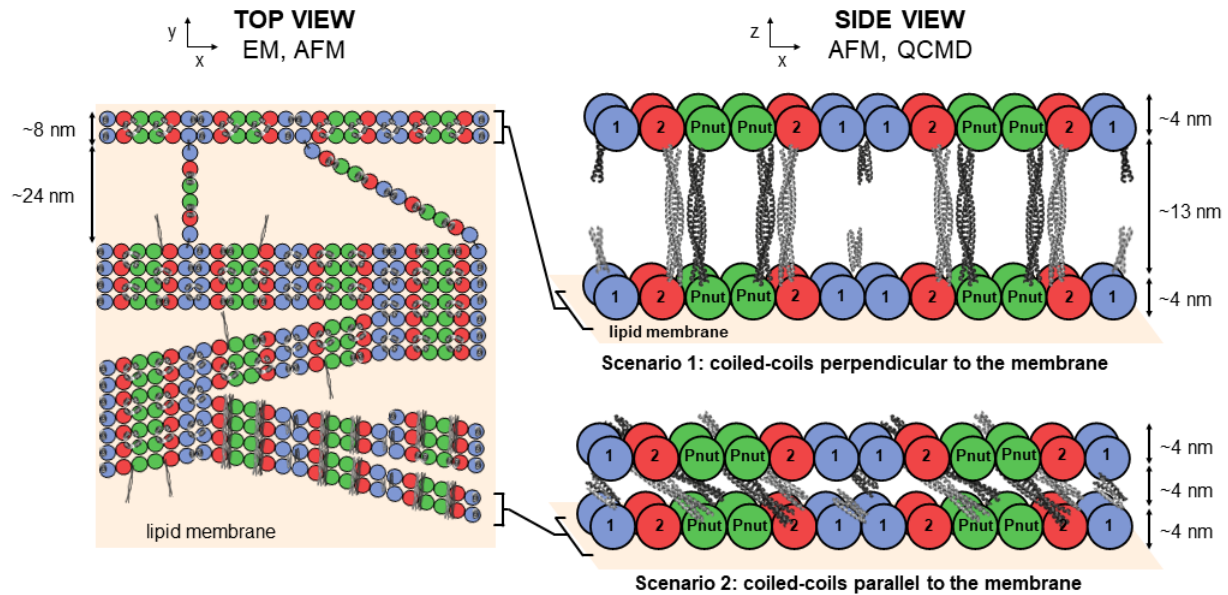
582 On the left in **Figure 9A**, we display the proposed organization of septin filaments in the
583 membrane-proximal layer. The hexamers form paired filaments by a combination of end-to-end
584 association mediated by homodimerization of the terminal DSep1 subunits of adjacent hexamers, and
585 lateral associations. Paired filaments in turn form in-plane bundles that occasionally branch and merge.
586 In addition, paired septin filaments are bridged by hexamers or dimers thereof. Similar bridges were
587 observed for yeast septin octamers on lipid monolayers, where the bridge length and spacing were also
588 multiples of the octamer length [57]. In the context of yeast septins, it was proposed that coiled coils
589 mediate septin filament pairing and the formation of perpendicular octamer cross-bridges between
590 paired filaments [9, 57]. The interpretation in that case was that at least some C-terminal extensions on
591 the terminal Cdc11 subunits of paired filaments were free to engage in interactions with C-terminal
592 extensions on the bridging filaments. We here propose that fly septins may similarly form bridges *via*
593 interactions of the C-terminal coiled coils of the terminal subunits (DSep1), facilitated by their
594 orientational flexibility conferred by the hinge regions. Indeed for the C-terminally truncated septin
595 hexamers, both pairing and cross-bridging are abrogated.

596 To explain why septins form multi-layers, we hypothesize that not all coiled coils are engaged in
597 interactions within the first membrane-proximal septin layer, thus leaving some coiled coils free to engage
598 off plane. Pairing of the septin filaments in the membrane plane is very tight, with a spacing of only 2 nm,
599 much smaller than the 13-nm extended length of the long coiled coils on Pnut and DSep2 subunits.
600 Similarly tight pairing was previously observed for yeast septins on lipid monolayers [57]. To explain this,
601 we consider that the coiled-coils may be oriented either upwards and engage transversely with coiled
602 coils on a second layer of septins (**Figure 8** right, scenario 1), or parallel to the membrane sandwiched

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603

(A) full-length septins



(B) Δ CC septins



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

between two layers of septins (**Figure 8** right, scenario 2). According to scenario 1, we would expect a layer height of 21 nm, which is larger than the maximum height of ~18 nm seen by AFM for dense yet sub-saturated membranes, and the average height of ~15 nm seen by QCM-D for saturated membranes. According to scenario 2, we would expect a layer height of ~12 nm, in better agreement with the typical

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625 heights seen in AFM. However, the AFM images show that the bundle heights are variable. We therefore
626 anticipate a combination of both scenarios, or even alternate scenarios, given the length and orientational
627 freedom of the coiled coils and the known ability of coiled coils to form both parallel and antiparallel
628 multimers [97, 98]. Importantly, layering is restricted to just two layers. Our interpretation is that coiled
629 coils are mostly engaged in in-plane associations while fewer are engaged in the recruitment of a new
630 layer of septins, so that with increasing layer height, there is a smaller and smaller probability of recruiting
631 additional material on top. This ultimately generates septin arrays that are very extensive in-plane, but
632 limited in thickness to a ~12 nm thick array with a layer of coiled coils sandwiched between two layers of
633 globular domains. Thicker layers appear to be rare, and may arise either from a different organization of
634 the second septin layer or from an additional septin layer.

635

636 **Biological implications**

637 Our observations of mechanically stable septin filament networks on model biomembranes suggest the
638 possibility that septins may directly enhance the cortical rigidity of cells by forming filaments on the inner
639 leaflet of the plasma membrane. With their high stability, septins could furthermore have an important
640 role in mechanical integration of the cell cortex, since the actin-myosin cortex in mesenchymal and
641 epithelial cells is known to turn over rapidly [99-102]. Although septins in cells are more dynamic than *in*
642 *vitro*, with typical *in vivo* half-times for fluorescence recovery on the order of 100 s (depending on septin
643 subunit), the turnover rate of septins is still slower than for actin (where the half-time for fluorescence
644 recovery is on the order of tens of seconds) [36]. In the context of amoeboid T-cell migration, it has for
645 instance been suggested that stable septin structures are important to ensure directional extension of
646 leading-edge protrusions [25]. New 3D-superresolution techniques [103] and EM tomography [13] of the
647 cell cortex may be able to resolve the proximity of cortical septins to the cell membrane to answer the
648 question whether septins indeed interact directly with the membrane at the cell cortex.

649

650 **Materials and Methods**

651

652 **Septin purification**

653 Chemicals were bought from Sigma unless indicated otherwise. Recombinant *Drosophila* septin hexamers
654 composed of DSep1 with an N-terminal His₆ tag, DSep2, and Pnut with a C-terminal Strep tag II
655 (WSHPQFEK, 1058 Da) were purified from *Escherichia coli* BL21(DE3) cells (Agilent Technologies) using a
656 two-tag affinity purification scheme to aid the isolation of full-length complexes as explained in ref. [70].
657 Using the same cloning strategy as for wild type complexes [62], a coiled-coil truncation mutant of fly
658 septin hexamers referred to as the Δ CC mutant was made with C-terminal truncations right after the end
659 of the α 6-helix (DSep1 Δ C56, DSep2 Δ C111, Pnut Δ C123). Cell cultures for wild type and Δ CC septins were
660 grown at 37°C and expression was induced with 0.5 mM isopropyl- β -D-1-thiogalactopyranoside (IPTG)
661 when the optical density at 600 nm (OD600) reached a value between 2 and 3. Fluorescently tagged septin
662 complexes were obtained by tagging DSep2 with monomeric enhanced green fluorescent protein (mEGFP)
663 on its N-terminus [62]. To ensure GFP-folding, cell cultures for GFP-labeled septins were grown at 37°C
664 until the OD600 reached 0.6-0.8 and expression was induced overnight at 17°C.

665 Cell pellets collected after centrifugation (10 min, 2800 $\times g$, 4°C) were lysed on ice with a tip
666 sonicator in *lysis buffer* (50 mM Tris-HCl pH 8, 500 mM KCl, 10 mM imidazole, 5 mM MgCl₂, 20 mM MgSO₄,
667 0.1 mM guanosine diphosphate (GDP), 5% glycerol) supplemented with 0.25 mg/mL lysozyme (5934-D,

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668 Euromedex), 1 mM phenylmethylsulfonyl fluoride (Sigma), Complete® protease inhibitor cocktail (Roche),
669 and 10 mg/L DNase I (Sigma). The clarified lysate obtained after centrifugation (30 min at 20000 × *g*, 4°C)
670 was first run on a streptavidin affinity column (StrepTrap® HP, GE Healthcare) equilibrated with *septin 1*
671 *buffer* (50 mM Tris-HCl pH 8, 300 mM KCl, 5 mM MgCl₂, 5% glycerol) by eluting with 2.5 mM d-
672 Desthiobiotin. The peak fractions were pooled and run on a nickel affinity column (HisTrap FF crude
673 column, GE Healthcare) equilibrated with *septin 2 buffer* (50 mM Tris-HCl pH 8, 500 mM KCl, 10 mM
674 imidazole, 5 mM MgCl₂, 5% glycerol). Pooled fractions from the elution peak were dialyzed against *storage*
675 *buffer* (50 mM Tris-HCl pH 8, 300 mM KCl, 5 mM MgCl₂, 5% glycerol, 5 mM dithiothreitol (DTT)) at 4°C
676 overnight and concentrated to ~3-5 mg/mL using Vivaspin 6 concentrators (Sartorius, 6 ml, 100 kDa cut-
677 off). Mammalian septin hexamers composed of mouse Sept2 (98.6% identical to human Sept2, differing
678 in 5 out of 361 residues, based on sequence alignment with the Clustal Omega from
679 <https://www.ebi.ac.uk/Tools/msa/clustalo/>) with an N-terminal His₆ tag, human Sept6, and human Sept7
680 with a C-terminal Strep tag II were purified in the same manner [41]. Budding yeast septin octamers
681 (composed of Cdc3, Cdc10, Cdc11 and His₆-Cdc12) were purified as described [9] and stored in 50 mM
682 Tris-HCl buffer (pH 8) supplemented with 300 mM KCl. Purified septin complexes were flash-frozen in
683 liquid nitrogen and stored at -80°C.

684

685 **Septin characterization**

686 The concentration of septin complexes was determined by measuring the optical absorbance of the
687 solutions at 280 nm with a UV-VIS spectrophotometer (Thermo Scientific, Nanodrop 2000) and subtracting
688 the contribution of scattering (typically ~2-3%) from absorbance measurements between 320-340 nm
689 [104]. We used extinction coefficients of 0.545 L × g⁻¹ × cm⁻¹ for full-length *Drosophila* septins, 0.584
690 L × g⁻¹ × cm⁻¹ for mEGFP-tagged septins, 0.645 L × g⁻¹ × cm⁻¹ for the ΔCC mutant, and 0.565 L × g⁻¹ × cm⁻¹
691 for the mammalian septins, with the values all calculated from the sequence [105]. Molar concentrations
692 were converted into weight concentrations using the calculated molecular weights of 306.9 kDa for full-
693 length untagged *Drosophila* septins, 361.6 kDa for mEGFP-tagged septins, 237.8 kDa for the ΔCC mutant,
694 and 285.7 kDa for the mammalian septins. The purity and integrity of the proteins was evaluated for each
695 batch by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) using 4-15% gradient gels
696 (Mini-PROTEAN® TGX® Precast Protein Gels, Bio-Rad) with a Precision Plus Protein® Kaleidoscope® (Bio-
697 Rad) protein standard (see **Figure 1 – figure supplement 1A,B** for full-length and ΔCC septins,
698 respectively). The length of the purified complexes (as a readout of the stability of the hexameric
699 arrangement) was evaluated by transmission electron microscopy (TEM). Solutions of septins diluted to
700 65 nM with a buffer containing 300 mM KCl, to prevent polymerization, were deposited on glow-
701 discharged carbon coated copper grids (CF300-Cu, Electron Microscopy Sciences) and incubated for at
702 least 30 min. The samples were negatively stained with 2% uranyl acetate (Nanoprobes, Yaphank, USA),
703 air-dried, and examined with a FEI Tecnai Spirit (120 kV) electron microscope (ThermoFisher, Waltham,
704 USA). We observed rod-shaped oligomers, mostly having the expected 24 nm length of a hexamer (**Figure**
705 **1 – figure supplement 1A,B**). We performed a total of 10 preparations of the full-length fly septins and
706 verified that the protein quality by SDS-PAGE and by TEM analysis was constant. We used 2 preparations
707 of C-terminally truncated fly septins, 1 preparation of yeast septins, and 1 preparation of mammalian
708 septins.

709

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

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

716

717 **Septin coiled-coil size prediction**

718 To identify the regions of the C-termini of fly and human septins that can adopt a coiled-coil conformation,
719 we employed the coiled-coil prediction algorithm COILS ([https://embnet.vital-
720 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
721 identical when we used the scoring matrix MTIDK (derived from myosins, paramyosins, tropomyosins,
722 intermediate filaments, desmosomal proteins and kinesins) or MTK (derived from the sequences of
723 myosins, tropomyosins and keratins [106]), and for both weighted and unweighted scans, indicating little
724 bias due to the high incidence of charged residues. **Figure 1A** displays the predicted structures for fly
725 septins starting right after the α 6-helix (ending in ...DRLAK for DSep1; ...RLEQ for DSep2; ...KLSE for Pnut).
726 For DSep1, the algorithm predicted a 28-residue-long coiled-coil (LGEKDR...AQMQR) with 99.8%
727 probability. An 86-residue-long C-terminal coiled-coil (QQTFEA..QQLATA) was predicted for DSep2 (47%
728 probability for the first 21 residues and 86-99.9% probability for the following 65 ones). An 86-residue-
729 long stretch in the C-terminus of Pnut (LTQMEE...HVTLEE) was further predicted to adopt a coiled-coil
730 conformation with >97.9% probability. **Figure 1B** shows the corresponding predictions for human septins
731 starting right after the α 6-helix (ending in ... RLKR for Sept2; ...KLEE for Sept6; ...KLAA for Sept7). The C-
732 terminus of hSept2 was predicted to form a 28-residue-long coiled-coil (LLEKEA...QMCMQG) with 90.2%
733 probability. The coiled-coil prediction for mSept2 is identical. An 86-residue-long stretch in the C-terminus
734 of Sept6 (QETYEA...TAAELL) was predicted to adopt a coiled-coil conformation with 74-100% probability.
735 An 86-residue-long C-terminal coiled-coil (LAQMEE..RILEQQ) was further predicted for Sept7 with 99.5-
736 100% probability. The coiled-coil predictions were fully in line with the α -helix prediction output of the
737 secondary structure prediction programs PHYRE2 and PSIPREDv4.0 [107, 108]. From the end of the α 6-
738 helix to the start of the predicted coiled-coils, there are stretches of 24, 15 and 15 residues for DSep1,
739 DSep2 and Pnut, respectively, and stretches of 15, 15 and 19 residues for Sept2, Sept6 and Sept7,
740 respectively, that are predicted to be unstructured. To depict septin coiled-coils, the crystal structures of
741 which have not yet been published, in **Figure 1C**, **Figure 1-Figure supplement 1A**, **Figure 7B** and **Figure 9**,
742 we have used the available crystal structures of coiled-coil dimers and tetramers from vimentin (PDB
743 3UF1) (coiled-coil side-views in the figures) and the early endosomal SNARE complex (PDB 2NPS) (coiled-
744 coil top-views in the figures). The molecular structure visualization program PyMOL was used for isolating
745 helical segments, and rotating and coloring the dimeric and tetrameric coiled-coil segments shown in the
746 images.

747

748 **Preparation of small unilamellar lipid vesicles**

749 Lipids were purchased from Avanti Polar Lipids (Birmingham, AL). The following lipids were bought and
750 stored at a concentration between 10 and 25 mg/mL in chloroform: 1,2-dioleoyl-*sn*-glycero-3-
751 phosphocholine (PC), 1,2-dioleoyl-*sn*-glycero-3-phospho-L-serine (PS), and 1,2-dioleoyl-*sn*-glycero-3-
752 phosphoethanolamine-N-(lissamine rhodamine B sulfonyl) (rhodamine-PE). Furthermore, 1,2-dioleoyl-*sn*-

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753 glycerol-3-phospho-(1'-myo-inositol-4',5'-bisphosphate) (ammonium salt) (PI(4,5)P₂) was bought in
754 powder form and stored as a 0.5 mg/mL solution in a chloroform:methanol:water mixture (20:9:1 volume
755 ratio). Small unilamellar vesicles (SUVs) were prepared by drying mixtures of PC/PS/rhodamine-DOPE
756 (79.7:20.0:0.3 molar ratio), PC/PI(4,5)P₂/rhodamine-PE (94.7:5.0:0.3), or PC/PS/PI(4,5)P₂/rhodamine-
757 DOPE (74.7:20.0:5.0:0.3) in glass vials using an air stream. PC/PS lipid mixtures were resuspended in a
758 filtered and degassed imidazole buffer of pH 7.4 (20 mM imidazole-HCl, 50 mM KCl, and 2 mM MgCl₂).
759 The total lipid concentration of the stock solutions was 0.5 mM for TIRF imaging and 2.5 mM for Atomic
760 Force Microscopy (AFM) and Quartz-Crystal Microbalance with Dissipation monitoring (QCM-D)
761 experiments. PI(4,5)P₂-containing lipid mixtures were resuspended at a total lipid concentration of 0.25-
762 0.5 mM in a 50 mM citrate buffer of pH 4.8 containing equimolar amounts of trisodium citrate and citric
763 acid, 50 mM KCl, and 0.1 mM ethylenediaminetetraacetic acid (EDTA). The acidic pH promotes the
764 formation of homogeneous and fluid bilayers by reducing the net charge on the head group of PI(4,5)P₂
765 from -4 to -3 [109]. SUVs for TIRF experiments were obtained by sonication with a tapered microtip
766 (Branson, USA; 3 mm diameter) for 30 min in pulsed mode (30 s on/30 s off at 10% amplitude). SUVs for
767 AFM and QCM-D experiments were obtained by exposing the lipid suspensions to 5 freeze/thaw cycles
768 followed by sonication with a microtip (FisherBrand; 2 mm diameter) for 30 min in pulsed mode (1 s on/1
769 s off at 30% amplitude) with ice cooling. The SUVs were centrifuged (15 min at 15000 × g) to remove any
770 titanium particles that might come off the sonicator tip. All vesicles were stored in the fridge. PI(4,5)P₂-
771 containing SUVs were used within 7 days, and all other vesicles within 30 days.

772

773 Fluorescence microscopy

774 Samples were prepared in home-made flow channels assembled from cleaned coverslips and microscope
775 slides that were rendered hydrophilic in base piranha (5% hydrogen peroxide, 5% ammonium hydroxide
776 in water) at 70°C. Flow channels were prepared by assembling a dried coverslip and microscope slide with
777 two parafilm spacers spaced apart by ~2 mm and fixed in place by melting on a hotplate at 120°C. Septins
778 were fluorescently labeled by co-polymerizing 90 mol-% untagged hexamers with 10 mol-% mEGFP-
779 tagged hexamers. Septin assembly on supported lipid bilayers (SLB) was performed on the microscope.
780 SLBs were first formed by flushing the SUV stock solution into the flow channels and allowing for SUV
781 rupture and spreading (10 to 15 min). Correct SLB formation was ascertained by checking that the
782 fluorescence signal was spatially uniform and, using fluorescence recovery after photobleaching
783 measurements, that the bilayer was fluid. Residual SUVs were removed by washing the SLBs with 5
784 channel volumes of septin *polymerization buffer* (20 mM imidazole pH 7.4, 1 mM DTT, 0.1 mM MgATP, 50
785 mM KCl and 2 mM MgCl₂), containing 2 mM trolox to suppress blinking [110] and a 2 mM protocatechuic
786 acid/0.1 μM protocatechuate 3,4-dioxygenase mixture to suppress photobleaching [111]. Septin
787 hexamers diluted to a concentration between 10 nM and 1 μM in *polymerization buffer* were then flushed
788 onto the SLBs and assembly was allowed to proceed for 30 minutes at 20°C. In case of net-neutral (pure
789 PC) bilayers, we had to include 0.1 wt-% methylcellulose in the buffer to crowd the septins close enough
790 to the surface to allow visualization by TIRF microscopy. In case of anionic bilayers, we did not use
791 methylcellulose.

792 TIRF imaging was performed with a Nikon Eclipse Ti-E inverted microscope equipped with a TI-
793 TIRF-E motorized TIRF Illuminator (Roper Scientific), a Nikon Apo TIRF 100×/1.49 N.A. oil immersion
794 objective, and a 491 nm/50 mW Calypso laser (Cobolt, Solna, Sweden). Images were acquired with a
795 QuantEM 512SC EMCCD camera (Photometrics, Roper Scientific, Tucson, AZ, USA) using an exposure time

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796 of 50 ms. The microscope was controlled with MetaMorph 7.5 software (Molecular Devices, Sunnyvale,
797 CA, USA). Images were processed (contrast enhancement and look-up table inversion) and analyzed
798 (integrated intensity calculations) with Fiji software [112]. Fluorescence recovery after photobleaching
799 was performed using a NikonA1 confocal microscope using a 100× oil immersion objective and an argon
800 laser (Coherent, CA, USA) at excitation wavelengths of 488 nm for septins and 561 nm for rhodamine-PE
801 lipids. The septins or the bilayer were first bleached by briefly (1 s) illuminating a circular region with a
802 radius of 5 μm using a high (70%) output laser power. The fluorescence recovery was then measured for
803 5-7 minutes by illuminating the sample using a low (0.1-2%) output laser power and 30 ms exposure time.
804 For the first 30 seconds after the bleach, images were acquired at 1 frame per second. For the remaining
805 acquisition time, images were acquired at a rate of 1 frame per 10 seconds.

806

Scanning transmission electron microscopy of septin bundles

808 To determine the morphology and mass per length (MPL) of septin bundles formed in solution, we
809 performed scanning transmission electron microscopy (STEM) using tobacco mosaic virus (TMV) rods with
810 a well-defined length (300 nm), width (18 nm) and MPL (131 kDa/nm) as an internal mass standard [113,
811 114]. Carbon or formvar+carbon coated copper grids (Ted Pella, Redding, CA, USA) were first incubated
812 for 30 s with 3 μL TMV (0.01-0.02 mg/ml dispersion in phosphate-buffered saline), a kind gift from Dr.
813 Jean-Luc Pellequer (Institut de Biologie Structurale, Grenoble, France), washed with ultrapure water, and
814 blotted with filter paper. Next, 5 μL of a solution of septins preassembled for 1 h at 20°C at a concentration
815 between 50 and 500 nM was deposited and left for 1 min. Finally, the grids were washed with ultrapure
816 water to remove excess salts and left to dry in air at 37°C. Images of 3072 \times 2207 pixels (16 bits) were
817 acquired at different magnifications (from 15000 to 50000 \times) and at an acceleration voltage of 10 to 20
818 kV, a current of 100 pA, and a pixel dwell time of 3 to 5 μs on a FEI Verios 460 STEM microscope. The MPL
819 was determined by analysis in Fiji [112] following a published procedure [114]. Briefly, we selected images
820 that contained both TMV rods and septin bundles. For intensity calibration, boxes with a width of 25 to
821 45 nm (depending on the image resolution) and a length of 100 nm were drawn along the TMV rods, to
822 obtain the protein signal, and on both sides of each rod, to obtain the background signal. The conversion
823 factor from intensity (in counts) to mass (in kDa) per pixel was determined from the integrated intensities
824 of at least three TMV rods per image. A similar procedure was then used to obtain the MPL of septin
825 bundles, drawing 100 nm long boxes over the bundles that encompassed their width (20 to 50 nm),
826 together with equal-sized boxes on either side of the bundle for background subtraction. The width of the
827 septin bundles was measured by drawing a line in Fiji perpendicularly across septin bundles and estimating
828 the distance between the edges by eye.

829

Transmission electron microscopy of septins on lipid monolayers

831 Samples for transmission electron microscopy (TEM) were prepared by incubating septin hexamers with
832 lipid monolayers formed at an air-buffer interface. Teflon wells were filled with 20 μL of a solution of 65
833 nM (fly) or 70 nM (mammalian) septin hexamers in a 50 mM Tris-HCl (pH 8) buffer with 50 mM KCl. A drop
834 (\sim 0.5 μL) of a 0.5 g/L lipid solution in chloroform was deposited at the air-water interface in each well to
835 form a lipid monolayer. The Teflon block was kept overnight at 4°C in a humid chamber, while stirring the
836 solution in each well with a magnetic stirrer bar. Lipid monolayers with adsorbed septins were collected
837 by briefly placing hydrophobic grids (Electron Microscopy Sciences, Hatfield, PA, USA) on the surface of
838 the solutions with the carbon-coated side facing the solution. Grids with the collected monolayers facing

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839 up were stained at once with 2% uranyl formate (Electron Microscopy Sciences) in water by depositing 4
840 μL on the grid and simultaneously blotting the excess solution. Samples were imaged using a Tecnai Spirit
841 electron microscope (Thermo Fischer) operated at an acceleration voltage of 120 V. We repeated the
842 experiments twice having each time 2 replicates.

843

Cryo-electron microscopy of septins on large unilamellar lipid vesicles

844 Samples for cryoEM were prepared by incubating septin hexamers with large unilamellar vesicles (LUVs).
845 Lipid mixtures at a total lipid content of 50 μg per sample in glass vials at the desired lipid ratios were
846 dried with argon and left in vacuum for 2 h to remove chloroform. LUVs were formed by hydrating the
847 lipids in high salt buffer at a concentration of 0.5 mg/mL (20 mM imidazole pH 7.4, 1 mM DTT, 300 mM
848 KCl and 2 mM MgCl_2). The vesicles were next incubated for 30 min with 5 μg of septins at a concentration
849 of 1.6 μM (0.5 g/L) in high salt septin storage buffer, corresponding to a lipid-to-protein weight ratio of
850 10:1. Meanwhile, lacey carbon coated grids (Electron Microscopy Science, EMS, France) were plasma
851 treated for 30 s to hydrophilize the surface and remove any impurities. Vesicles diluted in septin
852 *polymerization buffer* to a final concentration of 0.3 μM (0.1 g/L) were applied [78]d to the grid straight
853 after plasma treatment. Prior to the vitrification of the sample, 10 nm gold beads (EMS) were added in
854 solution to be subsequently used as fiducial markers. Excess liquid was blotted and the samples were
855 vitrified in liquid ethane using an automatic plunge freezer (EM PG, Leica, Wetzlar, Germany). Samples
856 were imaged using a FEI Tecnai G2 microscope equipped with a LaB6 filament and a 4KX 4K CMOS camera
857 F416 (TVIPS, Gauting, Germany) operated at an acceleration voltage of 200 kV and a magnification of
858 50000 \times . Data collection was carried out using the EMTool (TVIPS) software suite. Tilt series were acquired
859 from -60 to 60 degrees using a saxton angular data collection scheme. Individual images were collected
860 with 0.8 electrons per \AA^2 for a total dose of less than 70 electrons per \AA^2 . IMOD software [115] was
861 primarily used for data processing and alignment for individual images. The reconstructions were
862 performed using either IMOD (Weighted back projection) or Tomo3D (SIRT) [116]. The segmentation of
863 the volumes was performed manually using IMOD. We repeated the experiments three times.

864

Atomic force microscopy of septins on supported lipid bilayers

866 AFM samples were prepared on silicon substrates in order to approximate the conditions used for TIRF
867 imaging and QCM-D measurements. Silicon wafers (Prime Silicon, Les Ulis, France) were cut into squares
868 of $9 \times 9 \text{ mm}^2$, rinsed with absolute ethanol, blow-dried with N_2 gas, and treated with UV/ozone (Bioforce
869 Nanoscience, Ames, IA) for 30 min to remove organic contaminants and render the surface hydrophilic.
870 Each substrate was glued (Picodent Twinsil, Wipperfurth, Germany) on a 15-mm diameter metal disc (Agar
871 Scientific) covered with a hydrophobic protector film (Bytac surface protection laminate; Sigma). An SLB
872 was formed on the silicon wafer piece by incubating for 30 min with 100 μL of a 100 $\mu\text{g}/\text{mL}$ SUV solution
873 diluted in septin *polymerization buffer* with an enhanced KCl concentration to promote SUV rupture (20
874 mM imidazole pH 7.4, pH 7.4, 1 mM DTT, 150 mM KCl and 2 mM MgCl_2). Residual SUVs were washed off
875 with 1 mL of septin *polymerization buffer*. The SLB was kept hydrated by leaving 100 μL buffer on the
876 wafer surface and 50 μL of septin solution was added to reach a final concentration of either 12, 24 or 60
877 nM. After 15 min incubation at room temperature (19°C), unbound protein was washed off with 1 mL of
878 *polymerization buffer*. In most experiments, the samples were fixed for 1 minute with 1 wt-%
879 glutaraldehyde (GTA) in *polymerization buffer* and washed. Unfixed septins had a similar morphology as
880 fixed septins but were difficult to image at high resolution because they were easily disrupted by the
881

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882 scanning AFM tip. Reproducibility across biological replicates was checked by performing experiments on
883 5 protein preps.

884 AFM images were acquired at room temperature (23°C) with a Nanoscope Multimode 8 system
885 (Bruker, CA, USA) on samples immersed in *polymerization buffer*. Images of various lateral dimensions (5
886 to 20 µm) at a resolution of 512 × 512 pixels and a scan rate of 0.8 Hz were recorded with silicon cantilevers
887 (ScanAsyst-Fluid+; Bruker) with a nominal spring constant of 0.7 N/m and a sharp pyramidal tip with a
888 nominal radius of 2 nm and a maximal radius of 12 nm. Images were recorded in Peak Force Tapping mode
889 with a typical driving frequency of 2 kHz and tapping amplitude of 50 nm. Images were second-order
890 flattened using open source software Gwyddion [117] to correct for sample tilt and curvature of the xy
891 piezo scanner. Analysis of septin thread heights and widths was performed by constructing height profiles
892 (perpendicular to the thread axis, and averaging 5 pixels along the axis) in Gwyddion software. Heights
893 were defined as the peak value above the flat membrane surface. Widths were measured at a height of 1
894 nm below peak height. Sections across narrow threads generally showed a single peak, but wider threads
895 (> 30 nm in width) often showed multiple peaks. In the latter case, widths were measured at 1 nm below
896 the height of the two outermost peaks. We recall that tip convolution effects lead to an enlarged effective
897 width. Considering the expected tip shape, and the height at which the widths were measured, we expect
898 tip convolution to entail a width overestimation by between 2 and 10 nm.

899

900 QCM-D measurements of septin-membrane binding

901 To analyse the kinetics of septin binding and self-organisation on lipid membranes, we employed a surface
902 analytical technique known as quartz crystal microbalance with dissipation monitoring (QCM-D). This is
903 an acoustic technique that measures the adsorption of biomolecules onto the surface of a piezoelectric
904 quartz crystal that is cyclically sheared at its acoustic resonance frequency by applying an alternating
905 current [118]. The adsorption of molecules to the sensor surface results in a frequency shift Δf that, to a
906 first approximation, is proportional to the acoustic mass (including hydrodynamically coupled solvent) of
907 the adsorbed film [119] and a dissipation shift ΔD that provides information on the mechanical properties
908 of the film [85, 120].

909 We used silica-coated QCM-D sensors (QSX303, Biolin Scientific, Sweden) that were cleaned in an
910 aqueous solution of 2% sodium dodecyl sulfate (30 min), rinsed with ultrapure water, blow-dried with N₂
911 gas, and exposed to UV/ozone (BioForce Nanosciences, Ames, IA) for 30 min. The sensors were then
912 immediately mounted in the Q-Sense Flow Modules of the Q-Sense E4 system (Biolin Scientific) and
913 experiments were started within 15 min. Experiments were performed at a working temperature of 23°C.
914 The instrument was operated in flow mode, meaning that sample solution was continuously perfused
915 through the measurement chamber at a rate of 20 µL/min with a syringe pump (KD Scientific). We
916 sequentially incubated the sensors with SUVs to form a bilayer and then septins, interspersed by rinsing
917 steps. The surface was first equilibrated with *vesicle buffer* before flowing in the SUV suspension. The
918 adsorption of the SUVs onto the sensor surface and the subsequent formation of a supported lipid bilayer
919 (SLB) were monitored by measuring Δf and ΔD . The frequencies are measured in odd multiples
920 (overtones) of the fundamental resonance frequency of the piezoelectric sensor crystal. In our
921 experiments, the fundamental resonance frequency was 5 MHz and data were recorded at overtones $i =$
922 3 to 13. For further analysis, we selected data obtained at the 7th overtone, in view of its stable response
923 across all measurements. Other overtones provided qualitatively similar information and are thus not
924 shown. The frequency shifts were normalized according to $\Delta f = \Delta f_i / 7$. Once Δf and ΔD reached stable

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925 values, indicating that the SLB was ready, the surface was rinsed with *vesicle buffer* to remove residual
926 SUVs and then with septin *polymerization buffer* to equilibrate the ionic conditions. Next, the sensor was
927 perfused with septins in *polymerization buffer* at the desired concentration (60 or 100 nM) for at least 60
928 min. Finally, we tested the reversibility of septin-membrane binding by rinsing the sensor with
929 polymerization buffer. The experiments were performed at least in duplicate. Reproducibility across
930 biological replicates was checked by performing experiments on 5 protein preps.

931 QCM-D measurements can provide information on the thickness of the adsorbed septin films.
932 Provided that the septin layer is sufficiently uniform, dense and rigid, the Sauerbrey equation [119] relates
933 the frequency shift to the areal mass density of the film, $m_{\text{QCM-D}}$, which equals the layer density $\rho_{\text{QCM-D}}$
934 times the layer thickness $d_{\text{QCM-D}}$:

$$935 \quad \Delta f = -C^{-1} \times m_{\text{QCM-D}} = -C^{-1} \times \rho_{\text{QCM-D}} \times d_{\text{QCM-D}} \quad (1)$$

936 The mass sensitivity constant C is 18 ng/cm²/Hz for QCM-D sensors with a fundamental resonance
937 frequency of 5 MHz and the mass density for protein films is 1.1 g/cm³ to a good approximation [83]. For
938 sufficiently dense and rigid ($\Delta D / -\Delta f \ll 0.4 \times 10^{-6}$ /Hz) films, the film thickness $d_{\text{QCM-D}}$ is therefore
939 proportional to Δf with a proportionality constant of 6.1 Hz/nm.

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