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1 Title

2 Human septins in cells organize as octamer-based filaments mediating 3 actin-membrane anchoring

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35 Abstract

36 Septins are cytoskeletal proteins conserved from algae and protists to mammals. Septin
37 knock-out animals have established that septins are essential for animal physiology, but
38 their molecular function remains elusive. A unique feature of septins is their presence as
39 heteromeric complexes that polymerize into filaments in solution and on lipid membranes.
40 Although animal septins associate extensively with actin-based structures in cells,
41 whether actin-decorating septins organize as filaments and if septin organization impacts
42 septin function is not known. Customizing a tripartite split-GFP complementation assay
43 for probing the presence and composition of septin filaments *in situ* in cells, we show that
44 all septins decorating actin stress fibers are present as filaments whose integrity depends
45 on octameric septin protomers. Atomic force microscopy nanoindentation measurements

46 on cells confirmed that cell stiffness depends on the presence of octamer-containing
47 septin filaments. Super-resolution structured illumination microscopy revealed septin
48 fibers with widths compatible with their organization as paired septin filaments.
49 Nanometer-resolved distance measurements and single-protein tracking further showed
50 that actin-associated septin filaments are membrane-bound and largely immobilized.
51 Finally, reconstitution assays on supported lipid bilayers showed that septin filaments
52 mediate actin-membrane anchoring. We propose that septin organization as octamer-
53 based filaments is essential for septin function in anchoring and stabilizing actin fibers at
54 the plasma membrane.

55

56 **Introduction**

57 Septins comprise a family of cytoskeletal proteins conserved from algae and protists to
58 mammals (Cao et al., 2007; Momany et al., 2008; Nishihama et al., 2011; Pan et al.,
59 2007). Septins were discovered in budding yeast already 50 years ago (Hartwell, 1971;
60 Hartwell et al., 1970) as mutants that result in cytokinesis defects (Hartwell, 1971), and
61 follow-up studies in animal model systems established that they are also required for
62 animal cell division (Echard et al., 2004; Estey et al., 2010; Founounou et al., 2013;
63 Kechad et al., 2012; Kinoshita et al., 1997; Neufeld and Rubin, 1994; Surka et al., 2002).
64 However, septins are expressed in practically all human tissues, including non-dividing
65 neurons (Karlsson et al., 2021). There is compelling evidence that septins play roles in a
66 wide range of biological processes in non-dividing animal cells and tissues, including cell
67 motility, sperm integrity, neuron development, tissue morphogenesis, and host-pathogen
68 interactions (Fares et al., 1995; Finger et al., 2003; Gilden et al., 2012; Ihara et al., 2005;
69 Kim et al., 2010; Kissel et al., 2005; Kuo et al., 2012; Mostowy et al., 2010; Mostowy et
70 al., 2011; Nguyen et al., 2000; Shindo and Wallingford, 2014; Steels et al., 2007; Tada et
71 al., 2007; Tooley et al., 2009; Xie et al., 2007). The embryonic lethality of mouse and
72 *Drosophila* septin knock-outs (Adam et al., 2000; Fuchtbauer et al., 2011; Menon et al.,
73 2014; Roseler et al., 2011) emphasizes their essential contribution to animal physiology
74 and development, yet the precise molecular basis of their function in dividing and non-
75 dividing cells remains elusive.

76 Biochemical isolation of native septins from budding yeast, *Drosophila* and
77 mammalian cells and tissues revealed that septins exist as stable heteromeric complexes
78 that can polymerize into filaments and higher-order filament assemblies (Field et al.,
79 1996; Frazier et al., 1998; Hsu et al., 1998; Kim et al., 2011; Kinoshita et al., 2002; Sellin
80 et al., 2011). The isolation of recombinant septin complexes helped establish that septin
81 complexes are palindromes, with each septin in two copies and in a specific position
82 within the complex, with each monomer interacting with its neighbors by alternating
83 interfaces, named NC (from the N- and C-terminal domains) and G (from the GTP-binding
84 domain) (Bertin et al., 2008; DeRose et al., 2020; Farkasovsky et al., 2005; Garcia et al.,
85 2011; Huijbregts et al., 2009; Iv et al., 2021; John et al., 2007; Kinoshita et al., 2002;
86 Kumagai et al., 2019; Mavrakis et al., 2014; Mendonca et al., 2019; Rosa et al., 2020;

87 Sala et al., 2016; Sirajuddin et al., 2007; Soroor et al., 2021; Versele and Thorner, 2004).
88 Human septins are classified in four homology groups, namely the SEPT2 group (SEPT1,
89 2, 4, and 5), SEPT6 group (SEPT6, 8, 10, 11, and 14), SEPT7 group (SEPT7), and
90 SEPT3 group (SEPT3, 9, and 12) (Kinoshita, 2003). Cell-isolated human septins exist as
91 stable hexamers and octamers (Kim et al., 2011; Sellin et al., 2011; Sellin et al., 2014),
92 with hexamers composed of septins from the SEPT2, SEPT6, SEPT7 groups, and
93 octamers containing additional septins from the SEPT3 group (Fig. 1A). Whereas SEPT3
94 and SEPT12 are enriched in the brain and the testis, respectively, SEPT9 is expressed
95 in practically all human cell lines and tissues (Karlsson et al., 2021; Uhlen et al., 2015).

96 The feature of septins that led to the hypothesis that they make up the fourth
97 cytoskeleton element is the capacity of recombinant and cell-isolated septins to self-
98 assemble into filaments (Mostowy and Cossart, 2012; Valadares et al., 2017). The most
99 convincing evidence that septins form filaments *in vivo*, and that cell viability depends on
100 their ability to assemble into filaments, comes from electron microscopy and functional
101 data in budding yeast (Bertin et al., 2012; Byers and Goetsch, 1976; McMurray et al.,
102 2011; Ong et al., 2014; Rodal et al., 2005). The conservation of septins and the ability of
103 recombinant and cell-purified mammalian septin hexamers and octamers (hereafter
104 referred to as protomers) to self-assemble into filaments in solution and on lipid
105 membranes (DeRose et al., 2020; Iv et al., 2021; Leonardo et al., 2021; Soroor et al.,
106 2021; Szuba et al., 2021) has led to the assumption that human septins also organize as
107 filaments in cells, but formal evidence that this is the case is scarce. Immunogold electron
108 microscopy in mammalian cells has shown gold-decorated septins in close apposition to
109 cortical actin filaments and to the plasma membrane organizing in linear arrays (Hagiwara
110 et al., 2011; Kinoshita et al., 1997). Single septin protomers along actin filaments or the
111 membrane would, however, result in a similar pattern, as it would be the case for any
112 actin filament- or membrane-binding protein. It is reasonable to assume that septin rings
113 and fiber-looking segments that form in the cytoplasm of mammalian cells upon actin
114 depolymerization correspond to septin filaments or bundles thereof (Joo et al., 2007; Kim
115 et al., 2011; Kinoshita et al., 2002; Schmidt and Nichols, 2004). However, one cannot
116 exclude that septins on stress fibers are not filamentous, and that single septin protomers
117 on stress fibers spontaneously form filaments in the absence of stress fibers.
118 Furthermore, it was not shown that the observed septin fibers upon actin disassembly
119 originate from direct end-to-end septin complex polymerization. Whether all septins in
120 cells function as filaments, and how hexamers and octamers contribute to septin filament
121 formation and function are not known.

122 The fact that mammalian septins associate with membranes as well as with the
123 actin and microtubule cytoskeleton has made it difficult to dissect septin function, and has
124 at the same time naturally led to the speculation that septins mediate cytoskeleton-
125 membrane cross-talk (Dogterom and Koenderink, 2019). There is no doubt that septins
126 can associate both with actin and microtubules in cells (Bowen et al., 2011; Nagata et al.,
127 2004; Nagata et al., 2003; Spiliotis et al., 2008; Spiliotis et al., 2005; Surka et al., 2002;

128 Verdier-Pinard et al., 2017). The microtubule-binding domain on septins was identified
129 recently (Kuzmic et al., 2022), but actin-binding domains have not yet been identified
130 making it unclear if actin-septin binding involves direct interactions, or if such binding
131 occurs through myosin-II (Joo et al., 2007; Mostowy et al., 2010) or/and Borg proteins
132 (Calvo et al., 2015; Farrugia et al., 2020; Joberty et al., 2001; Liu et al., 2014; Salameh
133 et al., 2021). Similarly, although recombinant and cell-purified mammalian septins bind
134 lipid membranes in the absence of other physiological partners (Bridges et al., 2016; Dolat
135 and Spiliotis, 2016; Szuba et al., 2021; Tanaka-Takiguchi et al., 2009; Yamada et al.,
136 2016), whether there is direct septin-membrane binding in cells has not been formally
137 shown; the identification of the membrane-binding site of septins is a matter of debate
138 (Cavini et al., 2021). Septin-membrane association in dividing cells necessitates the
139 presence of Anillin, which itself binds the ingressing plasma membrane and recruits
140 septins to it (Field et al., 2005; Hickson and O'Farrell, 2008; Liu et al., 2012; Renshaw et
141 al., 2014). Given that Anillin is nuclear in interphase cells (Field and Alberts, 1995;
142 Oegema et al., 2000), it is not known if septin-decorated actin fibers and membranes in
143 non-dividing cells reflect membrane-bound septins.

144 To elucidate the interplay between human septin organization and function in non-
145 dividing cells, notably septin-actin-membrane interactions in cells, we used actin stress
146 fibers in U2OS cells as a model system. Septins in mammalian cells have been reported
147 to decorate stress fibers in multiple studies over the last 25 years (Calvo et al., 2015;
148 Connolly et al., 2011; Dolat et al., 2014; Joo et al., 2007; Kim et al., 2011; Kinoshita et al.,
149 2002; Kinoshita et al., 1997; Liu et al., 2014; Salameh et al., 2021; Schmidt and Nichols,
150 2004; Surka et al., 2002; Verdier-Pinard et al., 2017; Xie et al., 1999; Zhang et al., 1999).
151 Subsets of stress fibers are lost upon septin disruption or septin relocalization to
152 microtubules (Calvo et al., 2015; Kinoshita et al., 2002; Kuzmic et al., 2022; Salameh et
153 al., 2021; Schmidt and Nichols, 2004; Targa et al., 2019) suggesting an essential, yet still
154 unclear, role of septins in actin fiber formation or/and maintenance. To test if septins
155 organize as filaments in cells and determine septin filament composition, we combined a
156 tripartite split-GFP complementation assay with mutants disrupting specific septin-septin
157 interfaces in order to selectively perturb hexamers or octamers, or abolish polymerization
158 altogether. Atomic force microscopy nanoindentation measurements on cells were used
159 to assess the specific contribution of hexamers vs octamers to cell stiffness. We
160 employed super-resolution structured illumination microscopy to decipher the higher-
161 order assembly of septin filaments. Moreover, to determine whether septin filaments are
162 membrane-bound and if they have the capacity to bridge membrane-actin interactions,
163 we combined nanometer-resolved distance measurements and single protein tracking in
164 cells with cell-free reconstitution assays using supported lipid bilayers. Our findings
165 demonstrate that all actin-associated septins in cells organize as paired membrane-
166 bound filaments whose integrity and function depend on octamers.

167

168 **Results**

169 **Septins associate with contractile stress fibers.** Septins in mammalian cells have
170 been shown to localize to stress fibers (SFs) in multiple studies, but whether septins
171 associate preferentially with specific types of SFs and if septin organization differs among
172 SFs is not known. To answer these questions, we started by examining how septins
173 distribute with respect to the different types of SFs in U2OS cells, notably peripheral,
174 dorsal and ventral SFs, transverse arcs and the perinuclear actin cap (Fig. 1A). Given
175 that SFs are classified based on their subcellular localization and their anchoring at one
176 or both ends by focal adhesions (FAs) (Tojkander et al., 2012), we acquired confocal z-
177 stack images of cells co-stained for septins, actin filaments and the FA proteins, paxillin
178 or vinculin. We chose to examine the distribution of three septins expressed in U2OS
179 cells, namely SEPT2, SEPT7 and SEPT9 (Fig. 1B; Figs. S1, S2). SEPT2 and SEPT7 are
180 common to both hexamers and octamers, whereas SEPT9 is specific to octamers (Fig.
181 1A). Both septin immunostainings and imaging of septin-GFP fusions showed that the
182 distribution of all three septins with respect to SFs was identical. They all decorated
183 myosin-II containing contractile SFs (Fig. 1Bi-iii; Fig. S1Ai-ii, iv-v; Fig. S1Bi-vi), but not the
184 non-contractile dorsal ones (Fig. 1Biii,b; Fig. S1Aiii; Fig. S1Biv,a). Although septins
185 extensively decorated contractile SFs throughout their length, they were systematically
186 excluded from FAs (Fig. 1Bi,a; Fig. S1Ai,c; Fig. S1Bi,c; Fig.S2D). In addition to the
187 localization of septins to peripheral and ventral SFs, transverse arcs and perinuclear actin
188 caps, we also found septins associated with two types of actin nodes: geodesic actin
189 nodes on the ventral plasma membrane and actin nodes in transverse arcs (Fig. 1Biv;
190 Fig. S2A,C). Actin nodes were enriched in F-actin and α -actinin, while actin filaments
191 interconnecting actin nodes were decorated by septins and myosin-II in an aster-like
192 pattern.

193 U2OS cells express two SEPT9 isoforms, SEPT9_i1 and SEPT9_i3 (Kuzmic et al.,
194 2022), both of which are detected by our SEPT9 antibodies. The presence of SEPT9
195 showed that septin octamers are present on SFs, but does not exclude the possibility that
196 septin hexamers are also present. Furthermore, the diffraction-limited optical resolution
197 of our setup does not allow us to distinguish single septin protomers from septin filaments.
198 Septin decoration of SFs may therefore reflect the presence of either single protomers
199 (hexamers and/or octamers) or of filaments driven by hexamer or octamer polymerization,
200 or hexamer and octamer co-polymerization (Fig. 1A).

201
202 **Septins organize as filaments on contractile SFs.** Both hexamers and octamers have
203 an exposed SEPT2 NC interface at their termini (Fig. 1A) (Iv et al., 2021; Mendonca et
204 al., 2019; Soroor et al., 2021), thus detecting direct SEPT2-SEPT2 interactions would
205 provide a molecular readout of end-to-end septin polymerization of hexamers or/and
206 octamers driving the formation of septin filaments. To determine whether septins are
207 present as filaments, we designed a tripartite split-GFP complementation assay for
208 probing SEPT2-SEPT2 interactions *in situ* in living cells (Fig. 2A,B). This protein-protein
209 interaction assay involves the fusion of the proteins of interest to the two last beta-strands

210 of GFP, β 10 and β 11: in the presence of specific protein-protein interactions in cells
211 expressing GFP1-9 (GFP strands β 1- β 9), the GFP barrel is reconstituted leading to
212 fluorescence (Cabantous et al., 2013) (see methods and Fig. S3A-C for the design of the
213 assay). The implementation of the tripartite split-GFP complementation assay in budding
214 yeast for probing septin positioning was shown to measure intimate short-range physical
215 contacts and accurately reflected the spatial relationship among septin subunits within
216 the octamer (Finnigan et al., 2016). To probe SEPT2-SEPT2 interactions, we generated
217 β 10- and β 11-strand fusions with SEPT2 that we co-expressed using an inducible
218 bidirectional vector in U2OS cells constitutively expressing GFP1-9 (Fig. 2A; Fig. S3D).
219 The fluorescence from the reconstitution of the GFP barrel will occur only in the case of
220 specific SEPT2-SEPT2 interactions and will thus report the subcellular localization of
221 such interactions as a molecular signature of the presence of septin filaments.

222 To minimize the risk of not detecting SEPT2-SEPT2 interactions due to
223 endogenous untagged SEPT2 and given that the expression levels of SEPT2- β 10/ β 11
224 fusions were kept low to minimize overexpression artifacts (Fig. S3E), we consistently
225 knocked down endogenous SEPT2 in all subsequent experiments (Fig. S3F). Spinning
226 disk confocal imaging in live cells and in fixed cells co-stained for actin revealed the
227 presence of the reconstituted GFP (rGFP) on peripheral and ventral SFs, transverse arcs
228 and perinuclear actin caps (Fig. 2D,E), with the rGFP distribution closely resembling
229 endogenous SEPT2 immunostainings and SEPT2-GFP distribution (Fig. S1A). *In vitro*
230 reconstitution studies have shown that fly and mammalian septins organize both as single
231 and paired filaments (Szuba et al., 2021), prompting us to explore the origin of SEPT2-
232 SEPT2 rGFP on SFs. To this end, we generated septin protomer structure models, and
233 examined GFP complementation both from direct SEPT2-SEPT2 interactions within a
234 hexamer (Fig. 2B), and from SEPT2 facing another SEPT2 in apposed hexamers or
235 octamers of a paired septin filament (Fig. 2C). Examination of the distances and the
236 flexibility of the SEPT2 C-termini and the linkers in such models showed that GFP
237 reconstitution could originate either from direct SEPT2-SEPT2 interactions within one
238 hexamer (Fig. 2B) or from SEPT2 facing another SEPT2 in apposed hexamers or
239 octamers in a paired septin filament (Fig. 2C). Importantly, these models highlighted that
240 paired protomers would lead to GFP reconstitution whether the protomers polymerize or
241 not.

242 To test if SEPT2-SEPT2 rGFP on SFs originates from direct SEPT2-SEPT2
243 interactions, we designed a double point SEPT2 NC interface mutant (SEPT2 F20D,
244 V27D, hereafter SEPT2NCmut) to prevent end-to-end association and thereby abolish
245 polymerization (Fig. S4A) (Kuzmic et al., 2022; Sirajuddin et al., 2007). Reconstitution
246 assays using purified recombinant hexamers and octamers bearing these mutations
247 confirmed that this mutant abolishes septin polymerization, although it is still able to bind
248 actin filaments *in vitro* (Fig. S4B-E). Native PAGE in cell lysates expressing SEPT2 NC
249 interface mutants further confirmed that these mutants do not compromise protomer
250 integrity: the expression of either wild-type SEPT2 or SEPT2NCmut in SEPT2 knockdown

251 cells rescue equally well the hexamer and octamer distribution in control cells (Fig. 2F).
252 Strikingly enough, using this mutant in the context of the split SEPT2-SEPT2 assay
253 completely abolished SF localization as indicated by purely diffuse cytosolic fluorescence
254 (Fig. 2G,H) (hereafter referred to as "diffuse cytosolic"). Given that wild-type SEPT2-
255 SEPT2 rGFP was occasionally found as diffuse cytosolic, we quantified the distribution
256 of diffuse cytosolic and non-diffuse phenotypes in cells expressing wild-type SEPT2- vs
257 SEPT2NCmut- β 10/ β 11 fusions (see methods). While 95% of wild-type SEPT2-SEPT2
258 rGFP localized to SFs, 100% of SEPT2NCmut-SEPT2NCmut rGFP was diffuse cytosolic
259 (Fig. 2G,H). This result showed that direct end-to-end septin polymerization through an
260 intact SEPT2-SEPT2 NC interface is required for septin localization to SFs and thus that
261 septins on contractile SFs organize as single or paired septin filaments. We attribute the
262 fact that the split assay with the NC mutant still produced fluorescence to the plasticity of
263 septins which are able to use both NC and G interfaces when either one is compromised
264 (Kim et al., 2012). We speculate that SEPT2NCmut forms G-homodimers, in line with
265 earlier observations from other groups (Kim et al., 2012), thus enabling GFP
266 complementation.

267
268 **Single septin protomers do not associate with SFs.** The presence of septin filaments
269 does not exclude that single septin protomers are also present on SFs. To test if single
270 septin protomers associate with SFs, we examined the cellular distribution of the SEPT2
271 NC interface mutant fused to full-length GFP. Cells expressing this mutant exhibited a
272 diffuse cytosolic localization, demonstrating that this mutant does not bind SFs (Fig. 2I,J).
273 Given that wild-type SEPT2-GFP fusions also showed diffuse cytosolic localization in
274 addition to SF localization (Fig. S1A,vi) and to confirm that the diffuse cytosolic phenotype
275 was due to the incapacity of the NC mutant to bind SFs, we quantified the distribution of
276 diffuse cytosolic and non-diffuse phenotypes in cells expressing wild-type SEPT2-GFP
277 vs SEPT2NCmut-GFP fusions. While SEPT2-GFP was diffuse cytosolic in ~50% of cells,
278 100% of the cells expressing SEPT2NCmut showed this phenotype (Fig. 2I,J). This result
279 showed that single septin protomers in cells do not associate with SFs, meaning that all
280 septins decorating SFs are filamentous.

281
282 **SF-associated septin filaments contain predominantly octamers.** Our results
283 showed that all septins decorating SFs are filamentous but did not inform us on the
284 composition of septin filaments as SEPT2 is common to both hexamers and octamers
285 (Fig. 1A). Recombinant hexamers and SEPT9-containing octamers have the capacity to
286 co-polymerize *in vitro* (our own results in Fig. S5A and (Soroor et al., 2021)), but whether
287 this is the case in cells is not known. The presence of SEPT9 on SFs (Fig. S1B) and
288 native PAGE of cell lysates showing that all SEPT9 is incorporated in octamers (Fig. S5B)
289 suggest that septin filaments contain octamers. To explicitly visualize the presence of
290 octamers on SFs and to test if hexamers are also present there, we customized the
291 tripartite complementation assay for probing specifically SEPT7-SEPT9 and SEPT9-

292 SEPT9 as molecular signatures of octamers and SEPT7-SEPT7 as a molecular signature
293 for hexamers. Given that both SEPT9_i1 and SEPT9_i3 isoforms localize to SFs, we
294 probed the presence of octamers containing both these SEPT9 isoforms on SFs by
295 detecting both SEPT7-SEPT9_i1 and SEPT9_i1-SEPT9_i1 and SEPT7-SEPT9_i3 and
296 SEPT9_i3-SEPT9_i3 interactions (Fig. 3A,B).

297 Expression levels of all β 10/ β 11 fusions were kept low to minimize overexpression
298 artifacts (Fig. S3E), and endogenous SEPT7 and SEPT9 were consistently knocked down
299 in all subsequent experiments (Fig. S3G,H). As expected, rGFP from SEPT9_i3-
300 SEPT9_i3 localized to contractile SFs (Fig. 3C), similarly to SEPT9 immunostainings (Fig.
301 S1B), confirming that septin filaments contain SEPT9_i3-octamers. Split assays probing
302 SEPT7-SEPT9_i3 interactions entirely recapitulated these findings (Fig. 3D), with rGFP
303 additionally labeling cytoplasmic rings of $\sim 0.9 \mu\text{m}$ in diameter (Fig. 3Dii; Fig. S6C). To
304 confirm that SF-localized rGFP from SEPT7-SEPT9_i3 and SEPT9_i3-SEPT9_i3 depend
305 on the integrity of these interfaces and thus reflect direct SEPT7-SEPT9_i3 and
306 SEPT9_i3-SEPT9_i3 interactions, we designed a double point SEPT9_i3 NC interface
307 mutant (SEPT9_i3 M263D, I270D, hereafter SEPT9_i3NCmut) (Fig. S4A), a double point
308 SEPT9_i3 G interface mutant (SEPT9_i3 W502A, H512D, hereafter SEPT9_i3Gmut)
309 (Fig. S4A) and a double point SEPT7 G interface mutant (SEPT7 W269A, H279D,
310 hereafter SEPT7Gmut1) (Fig. S4A) (Kuzmic et al., 2022; Sirajuddin et al., 2007; Zent et
311 al., 2011). Native PAGE of these mutants with full-length GFP fusions confirmed that
312 SEPT9_i3NCmut completely disrupts octamers (Fig. S5B), whereas SEPT7Gmut1
313 completely disrupts octamers and hexamers (Fig. S7B). Split assays using these mutants
314 to disrupt the SEPT9_i3-SEPT9_i3 NC interface or the SEPT7-SEPT9_i3 G interface,
315 completely abolished SF localization (Fig. 3E; Fig. S5C-E; Fig. 3F; Fig. S6A,G),
316 confirming that SF localization requires intact SEPT7-SEPT9_i3 and SEPT9_i3-
317 SEPT9_i3 interfaces. All above assays and mutants gave identical results for SEPT9_i1,
318 confirming the presence of both SEPT9_i1- and SEPT9_i3-containing octamers in SF-
319 associated septin filaments (Fig. S5B,F-G; Figs. S6Di,ii and S6G).

320 By contrast, rGFP from SEPT7-SEPT7 interactions was unexpectedly difficult to
321 detect: although it localized to SFs (Fig. 4Ai, Bi), the majority was found on ectopic short,
322 needle-like bundles (Fig. 4Aii, Bii), similar to the localization of full-length GFP-SEPT7
323 fusions (Fig. 4C; Fig. S7C). These ectopic bundles did not localize to SFs (Fig. 4Aii; Fig.
324 S7C) and contained SEPT2 but not SEPT9 (Fig. 4C). These bundles thus most likely
325 consist of hexamers, in line with the capacity of recombinant hexamers to form septin
326 filament bundles *in vitro* (DeRose et al., 2020; Iv et al., 2021; Kinoshita et al., 2002;
327 Leonardo et al., 2021). The presence of rGFP on the ectopic bundles thus showed that
328 the split SEPT7-SEPT7 assay readily detects SEPT7-SEPT7 interactions originating from
329 hexamers. To explore the origin of SEPT7-SEPT7 rGFP on SFs, we generated structure
330 models of septin protomers in order to examine GFP complementation from SEPT7-
331 SEPT7 interactions within one hexamer (Fig. 4D), as well as from SEPT7 facing another
332 SEPT7 in apposed hexamers or octamers in a paired septin filament (Fig. 4E).

333 Examination of the distances and the flexibility of the SEPT7 N-termini and the linkers in
334 the structure models showed that GFP reconstitution can occur in the context of both
335 single and paired filaments. Thus SEPT7-SEPT7 rGFP on SFs could originate either from
336 SEPT7-SEPT7 interactions within a hexamer (Fig. 4D) or from SEPT7-SEPT7 across
337 paired hexamers or octamers (Fig. 4E).

338 An observation that could explain the difficulty to detect SEPT7-SEPT7 on SFs
339 was the dependence of SEPT7 localization on SEPT9 expression levels. We consistently
340 detected ectopic bundles when we exogenously expressed only SEPT7, either GFP-
341 SEPT7 or split SEPT7-SEPT7 (Fig. 4A-C; Fig. S7C), but not when we co-expressed
342 SEPT9 (Fig. 4F,G). We reasoned that in the absence of exogenous SEPT9, the slightest
343 excess of SEPT7 leads to ectopic hexamer-based bundles, also reducing the availability
344 of SEPT7 for forming octamers to bind SFs. SEPT7-SEPT7 interfaces being more stable
345 than SEPT7-SEPT9 ones (Rosa et al., 2020) would facilitate this process. Exogenous co-
346 expression of SEPT9, on the other hand, would cause incorporation of the exogenous
347 SEPT7 into octamers, thus preventing the formation of ectopic hexamer bundles.
348 Consistent with this hypothesis, SEPT7-SEPT7 rGFP was readily detectable on SFs
349 under conditions of exogenous SEPT9 co-expression (Fig. 4F; Fig. S7H). Furthermore, it
350 was difficult to find SEPT9-decorated SFs in cells also displaying ectopic hexamer-based
351 bundles (Fig. 4G,a). These observations raised the possibility that septin filaments on
352 SFs contain mostly, if not exclusively, octamers.

353 To identify the sources of the SEPT7-SEPT7 rGFP signal on SFs, we aimed at
354 perturbing hexamers while preserving octamers. To this end, we generated a single point
355 SEPT7 G interface mutant (SEPT7 H279D, hereafter SEPT7Gmut2) (Fig. S4A) that we
356 reasoned should destabilize the SEPT7-SEPT7 G-interface when present in both SEPT7
357 subunits, but preserve the SEPT7-SEPT9 G-interface if SEPT7 is mutated but SEPT9 is
358 wild-type. In line with these predictions, native PAGE showed that octamers are not
359 affected by the expression of SEPT7Gmut2 (Fig. S7B), and rGFP from SEPT7Gmut2-
360 SEPT9_i3 readily recapitulated normal septin localization on SFs (Fig. 4H; Fig. S6B,G).
361 Importantly, rGFP from SEPT7Gmut2-SEPT7Gmut2 localized to SFs but did not show
362 any ectopic bundles (Fig. 4I; Fig. S7G). The absence of ectopic hexamer bundles implies
363 that SEPT7Gmut2 completely abolished SEPT7-SEPT7 interactions within hexamers in
364 the bundles. We thus reasoned that the SF-localized rGFP from SEPT7Gmut2-
365 SEPT7Gmut2 originates from paired octamers (Fig. 4E). We cannot exclude that the
366 rGFP signal could originate from SEPT7Gmut2-SEPT7Gmut2 across paired hexamers
367 containing wild-type SEPT7 and SEPT7Gmut2 subunits next to each other, but we deem
368 this scenario highly unlikely: endogenous wild-type SEPT7 is largely knocked down (Fig.
369 S3H), and any remaining wild-type SEPT7 would have to interact with SEPT7- β 10Gmut2
370 that would also need to encounter SEPT7- β 11Gmut2 in an apposed hexamer within a
371 septin filament pair.

372 These observations altogether strongly indicate that the detected rGFP from
373 SEPT7-SEPT7 on SFs originates from paired octamers. Split assays using the

374 SEPT7Gmut1 mutant resulted in diffuse cytosolic distributions (Fig. S6A,G; Fig. S7A,E),
375 confirming that SF localization requires intact SEPT7 G interfaces. These results thus
376 support a scenario whereby septin filaments contain mostly, or even exclusively,
377 octamers. These findings further suggest that septins organize as paired filaments, or
378 bundles thereof.

379

380 **Septin octamers, but not hexamers, are essential for the integrity and function of**
381 **SF-associated septin filaments.** To further test the contribution of octamers and
382 hexamers to septin filament formation, we examined septin filaments under three
383 conditions: (a) the presence of hexamers and octamers (control condition), (b) the
384 absence of octamers, by knocking down SEPT9, and (c) the presence of octamers only,
385 by expressing SEPT7Gmut2 to disrupt hexamers while preserving octamers. As a
386 readout of septin filaments, we examined rGFP from SEPT2-SEPT2 in live cells while at
387 the same time imaging stress fibers (Fig. 5A-C). To assess the effects of the
388 perturbations, we quantified the distribution of non-diffuse (SF-associated and punctate)
389 vs. diffuse cytosolic phenotypes and calculated Pearson and Manders correlation
390 coefficients for actin-septin co-localization in all three conditions (Fig. 5D,E). Strikingly,
391 removing octamers by knocking down SEPT9 entirely removed the SEPT2-SEPT2 rGFP
392 signal from all SFs, leaving behind a punctate pattern not localizing to SFs, suggesting
393 that filamentous septin integrity depends entirely on octamers. On the other hand,
394 preserving octamers in the absence of hexamers, through the expression of
395 SEPT7Gmut2, preserved septin filament localization to SFs, showing that the absence of
396 hexamers does not compromise septin filament integrity. Altogether, these results
397 strongly support that septin filaments contain mostly, or even exclusively, octamers, with
398 their integrity depending on octamers but not on hexamers.

399 To question the functional contribution of hexamers vs octamers in our cells, we
400 turned to atomic force microscopy (AFM) nanoindentation for measuring cell stiffness.
401 The principle of a nanoindentation experiment is to physically indent a cell with an AFM
402 cantilever tip, measure the applied force, and fit the experimental force-indentation curves
403 to a viscoelastic model in order to extract the elastic modulus (E_0) and the fluidity (β) of
404 the cell (Fig. 5F, see methods). Septin depletion has been previously shown to reduce
405 cell stiffness, using AFM, in cultured mammalian cells (Mostowy et al., 2011), but the
406 specific contribution of hexamers vs octamers to cell mechanics was not explored. To
407 determine the contribution of hexamers vs octamers to cell stiffness, we compared
408 nanoindentation measurements under the same conditions as earlier: (a) the presence of
409 hexamers and octamers (control condition), (b) the absence of octamers, by knocking
410 down SEPT9, and (c) the presence of octamers only, by expressing SEPT7Gmut2 (Fig.
411 5A-C). Measurements were made on cells plated on Y shape-micropatterned substrates
412 in order to minimize variability due to size and shape differences among cells (Rigato et
413 al., 2015). While removing hexamers did not have any effect, the depletion of octamers
414 resulted in a statistically significant decrease in cell stiffness and a corresponding

415 increase in cell fluidity (Fig. 5F). We conclude that octamers are essential not only for the
416 integrity of SF-associated septin filaments, but also for their function. These data also
417 further support the scenario that septin filaments contain mostly, or even exclusively,
418 octamers.

419

420 **Super-resolution microscopy reveals septin fibers running longitudinally along**
421 **and around SFs and interconnecting SFs.** Having shown that all septins associated
422 with SFs are filamentous, we naturally aimed at visualizing how septin filaments organize
423 in cells on the different types of SFs. To this end, we employed super-resolution structured
424 illumination (SIM) microscopy in cells co-stained for SEPT7 (as a pan-septin filament
425 marker), actin filaments, and α -actinin or myosin-II heavy chain (MHCA). We examined
426 septin filament organization on perinuclear actin caps, transverse arcs, including at arc
427 nodes, on ventral SFs and at ventral actin nodes (Fig. 6A-E). Regardless of the type of
428 SFs that septins associated with, we noticed that septin filament morphology was very
429 different from that of the actin filament bundles in SFs. While actin filament bundles
430 typically appeared as straight, rigid fibers throughout the cell and for different SF types,
431 septin fibers consistently appeared less straight and with lower orientational persistence
432 (*Note: we choose to use septin "fibers" instead of septin "filaments" in this section to avoid*
433 *confusion with single or paired septin filaments or bundles thereof; we discuss the*
434 *composition of septin fibers below*). Unlike core SF components like myosin and the actin
435 crosslinker, α -actinin, which displayed a sarcomere-like punctate distribution (Fig. 6E,
436 MHCA), septin fibers were distinctly separate from SFs, organizing in three manners: (a)
437 septin fibers running longitudinally along SFs, either on the side of SFs with their signal
438 segregated from the F-actin signal, or overlapping with SFs with the septin and F-actin
439 signals merging (Fig. 6Aa'; Ei,e'; Eiii,f,g'), (b) septin fibers running longitudinally along
440 SFs and diagonally across their width, as if wrapping around the SFs (Fig. 6Ab'; Eii,h',i'),
441 and (c) septin fibers running longitudinally along segments of SFs while interconnecting
442 different SFs and also forming connections to other septin fibers, thus forming a fiber
443 meshwork (Fig. 6Cc'; Dd'; Eiv,k'). When septin fibers connected SFs, the septin fiber
444 segments in between SFs frequently colocalized with F-actin signal, but in many
445 instances there was no detectable F-actin signal along these segments.

446 Regardless of the type of SFs, the majority of septin fibers appeared thinner than
447 their associated SFs. We noticed that septin fibers were often thicker on the SF segments
448 adjacent to FAs (Fig. 6Eiii,g'; Eiv,j'), but thicker septin fibers were occasionally also found
449 on arcs, caps and ventral SFs. These thicker septin fibers did not exceed the width of the
450 associated SF, and appeared either as single thick fibers, or what looked like two closely-
451 apposed thin fibers (dashed rectangle in the SEPT7 channel of Fig. 6Eiii). To compare
452 septin fiber thicknesses across the different SF types we measured the width of septin
453 fibers at multiple positions along their length and in multiple septin fibers for each SF type.
454 The full width at half maximum (FWHM) was calculated from the fluorescence intensity
455 profiles of the measured widths (Fig. 6F). Thick septin fiber widths from ventral SFs,

456 where thicker septin fibers were found more frequently, were plotted separately. The
457 comparison of the width distributions showed that all thin septin fiber populations were
458 very similar to each other, with median FWHM values in the range of 123-137 nm across
459 the different SF types, and distinct from the thick ones that showed an almost 2-fold higher
460 median FWHM value of 231 nm (Fig. 6F,H). There was no statistically significant
461 difference between thin septin fiber widths on caps, asters, arcs and ventral SFs (Fig.
462 6F).

463 In an effort to determine whether the septin fibers are single or paired septin
464 filaments ("double septin filaments"), or bundles thereof, we compared the width values
465 with the widths of microtubules in the same cells. FWHM values of single microtubules
466 (MTs), which are 25-nm wide tubes, are routinely used as the gold standard for assessing
467 the performance of super-resolution microscopy techniques. MTs were stained using
468 whole primary and fluorophore-coupled secondary IgG antibodies, just like for septin
469 stainings, leading to an estimated real MT width of ~60 nm (Fig. 6I) (Weber et al., 1978).
470 Measurements of MT widths in our cells with SIM resulted in an average FWHM value of
471 115 nm, in line with reported FWHM values for MTs by SIM (Hamel et al., 2014; Wegel
472 et al., 2016) given that the lateral resolution of SIM is roughly half of the diffraction limit,
473 i.e., ~110 nm. Given that the observed size in our images is the convolution of the real
474 object size with the point spread function (PSF) of the SIM microscope, we simulated the
475 predicted image size as a function of the real fiber size (Fig. 6G) (see methods). The
476 comparison of the estimated real widths of primary and secondary IgG-decorated septins,
477 assumed to organize as single or as paired filaments with either narrow (~5 nm) or wide
478 (~20 nm) spacing (Leonardo et al., 2021) (Fig. 6I), with the widths predicted from our
479 FWHM measurements of immunostained septins (Fig. 6H), suggests that the thin septin
480 fiber widths are compatible with single or paired septin filaments, whereas the thick septin
481 fibers could correspond to two single or two double septin filaments.

482 We also wondered about the length of the SF-associated septin fibers. Although
483 our results show that all septins are filamentous, the lateral resolution limit of SIM did not
484 allow us to distinguish if what appears as continuous fiber signal originates from a single
485 fiber or from adjacent fibers partially overlapping at their ends. The uniform thickness
486 across thin septin fibers let us speculate that these could correspond to single septin
487 fibers. Length measurements of presumably single septin fibers showed that short septin
488 fibers associated with and interconnecting actin nodes were on the order of 0.5-3.5 μm in
489 length, whereas septin fibers on arcs, actin caps and ventral SFs were as short as 0.5-1
490 μm in length and as long as 10-15 μm (Fig. 6J).

491
492 **SF-associated septin filaments are closely apposed to the plasma membrane.**
493 Having shown that all SF-associated septins are filamentous and given the extensive,
494 intimate association of septin filaments with actin fibers observed by SIM, we wondered
495 how septin function relates to septins being filamentous. Recombinant human septins can
496 bind and cross-link actin filaments, but can also bind lipid membranes, raising the

497 hypothesis that septin filaments in cells anchor SFs to the plasma membrane. Although
498 it is often assumed that human septins are found at the plasma membrane, there is no
499 formal proof of direct septin-membrane binding in cells. Mutants of the putative
500 membrane-binding polybasic stretch of residues in the septin $\alpha 0$ helices (Cavini et al.,
501 2021; Zhang et al., 1999) also disrupt the respective NC interfaces and thus the integrity
502 of the protomers (Bertin et al., 2010; Kuzmic et al., 2022), and thus do not allow us to
503 conclude on direct septin-membrane interactions. A first indication that SF-associated
504 septins might be membrane-bound came from live cell extraction experiments. Short
505 (30s-1 min) incubations of live cells with low concentrations of Triton X-100 detergent
506 right before fixation is routinely used for removing cytosolic pools of cytoskeletal proteins,
507 for example myosin, while preserving pools that are stably associated with the actin
508 cytoskeleton, with the aim to reduce diffuse cytosolic signal and enhance the signal on
509 SFs to reveal better its sarcomere-like distribution. Strikingly, while extracting the plasma
510 membrane after fixation entirely preserved septin localization to SFs, live-cell extraction
511 removed septins from all SFs while preserving actin, myosin and the actin crosslinker, α -
512 actinin, on SFs (Fig. 7A, data not shown for α -actinin). Septins thus did not seem to
513 behave like core components of SFs such as myosin and actin crosslinkers, and their
514 sensitivity to the detergent suggested that they might be bound to the membrane.

515 To directly test if septins on SFs are close to the plasma membrane, we employed
516 a metal-induced energy transfer (MIET) assay in cells (Chizhik et al., 2014). In MIET, the
517 fluorescence lifetime is dependent on the distance of fluorophores from a metal layer,
518 allowing us to use fluorophore lifetime measurements for deducing the axial distance of
519 fluorophores from a gold-coated coverslip surface with an axial resolution of a few
520 nanometers (Fig. 7B). We hypothesized that septins could either associate with the
521 plasma membrane while interacting with SFs, or that septins interact with SFs in the
522 absence of any septin-membrane association. To distinguish these scenarios, we
523 compared distances of the fluorescent protein, mApple, in three conditions: (a) mApple
524 N-terminally fused to the 20 N-terminal residues of neuromodulin/GAP43 that contains
525 palmitoylated cysteines and functions as a membrane targeting signal (GAP43-mApple);
526 this fusion thus served as a reference for fluorophores localizing directly at the plasma
527 membrane, (b) SEPT9_i3-mApple as a reference for ventral SF-associated septin
528 octamers, and (c) SEPT9_i3-mApple-CAAX as a reference for septins targeted to the
529 plasma membrane through the H-Ras CAAX motif, i.e. the 20 C-terminal residues of H-
530 Ras that contain palmitoylated and farnesylated cysteines and function as a membrane
531 targeting signal. Representative lifetime decay traces are shown in Fig. 7C and Fig.
532 S8A,B. Strikingly, the distance of mApple from the metal surface, derived from the
533 lifetime-distance dependence curve (Fig. 7D and methods), was the same for SF-
534 associated septins, membrane-bound mApple, and membrane-bound septins, strongly
535 indicating that septins are closely apposed to the plasma membrane (Fig. 7E). Lifetime
536 measurements of AF568-phalloidin bound to ventral SFs under the same conditions
537 placed SFs significantly further away, by ~ 25 nm, from the plasma membrane (Fig. 7E).

538 MIET assays being limited to probing interactions within 200 nm from the metal surface,
539 it was not feasible to probe septin populations on SFs localized further away, notably
540 transverse arcs and perinuclear actin caps. Given that arcs and actin caps are most likely
541 coupled to the dorsal plasma membrane (Burnette et al., 2014; Maninova et al., 2017), it
542 is conceivable that septins associated with these SF pools are also membrane-bound.

543
544 **Septin filaments anchor actin filaments to lipid membranes.** Since SF-associated
545 septins are closely apposed to the membrane, we naturally wondered if septin filaments
546 could function to anchor stress fibers to the plasma membrane. In the absence of
547 available septin membrane-binding and actin-binding mutants, we turned to reconstitution
548 assays on supported lipid bilayers (SLBs), comparing phosphatidylcholine- (PC) vs
549 phosphatidylinositol(4,5)-bisphosphate (PI(4,5)P₂)-containing membranes (Fig. 7F; Fig.
550 S8C), PI(4,5)P₂ being a septin-interacting lipid (Szuba et al., 2021). To image only truly
551 membrane-associated structures, we used total internal reflection fluorescence (TIRF)
552 microscopy in the absence of crowding agents. Actin filaments alone did not bind lipid
553 membranes, whereas septin octamers alone specifically bound PI(4,5)P₂-containing
554 membranes (data not shown), in line with previous reports for mammalian septin
555 hexamers (Szuba et al., 2021). To test if septins can anchor actin to membranes, we
556 either preformed actin-septin bundles in solution and then added them to SLBs, or co-
557 polymerized septins and actin on SLBs. In both cases, and specifically on PI(4,5)P₂-
558 containing membranes but not on membranes composed of PC, actin filaments and actin
559 filament bundles were anchored to the lipid bilayers (Fig. 7F; Fig. S8C), showing that
560 septin filaments can indeed at the same time bind membranes and actin and thus mediate
561 membrane-actin anchoring.

562
563 **Single protein tracking reveals that septins are immobilized on actin stress fibers.**
564 Since septin filaments are able to anchor actin filaments to lipid membranes in vitro and
565 given that SF-associated septins are in close proximity to the plasma membrane, we
566 questioned whether the molecular dynamics of septins at the plasma membrane could
567 reveal the potential SF-anchoring function of septins in living cells.

568 To determine the molecular behavior of septins in the vicinity of the plasma
569 membrane and actin stress fibers, we combined photoactivated localization microscopy
570 (PALM) with live-cell single protein tracking of SEPT9_i3 and actin fused to
571 photoswitchable mEos fluorescent proteins (mEos3.2 and mEos2, respectively) using
572 sptPALM (Manley et al., 2008; Rossier et al., 2012). Cells were co-transfected with mEos-
573 fused proteins and GFP-paxillin as a FA reporter or GFP-actin as a SF and FA reporter.
574 In brief, this method uses TIRF microscopy to detect and track numerous sparse photo-
575 activated proteins of interest within 200 nm above the coverslip surface at high-frequency
576 (50Hz acquisition), allowing us to reconstruct thousands of protein trajectories (Fig.
577 7G,H,I). For trajectories lasting at least 260 ms, we compute the mean square
578 displacement (MSD), which describes the diffusion properties of a molecule. We then sort

579 trajectories according to their diffusion modes (immobile, confined, free-diffusive; Fig. 7K),
580 and extract diffusion coefficients (D_{diff} , D_{conf}) (Fig. 7J; Fig. S8D,E; see methods). We first
581 looked at the dynamic behavior of mEos2-actin in SFs labelled with GFP-actin (Fig. 7G).
582 mEos2-actin was found inside FAs and also linearly organised along SFs between FAs,
583 as expected. Actin mostly displayed immobilized and confined behaviors, as illustrated
584 by the large fractions of immobilization and confined diffusion (Fig. 7G,K; immobile: 88.5
585 $\pm 0.5\%$, confined: $6.8 \pm 0.3\%$, mean \pm SEM) and a distribution of diffusion coefficients
586 centered around 1.5 - $2.5 \cdot 10^{-3} \mu\text{m}^2 \cdot \text{s}^{-1}$ (Fig. 7J). In line with septin immunostainings (Fig.
587 1Bi,a; Fig. S1Ai,c; Fig. S1Bi,c; Fig.S2D), single SEPT9_i3-mEos3.2 molecules were
588 rarely found inside FAs, but were linearly organized between FAs decorating SFs (Fig.
589 7H). Like actin, also SEPT9_i3-mEos3.2 was found to be primarily immobilized and
590 confined (Fig. 7H,K; immobile: $70.0 \pm 1.9\%$, confined: $13.9 \pm 0.8\%$, mean \pm SEM).
591 Contrary to actin, however, SEPT9_i3-mEos3.2 also displayed a significant freely
592 diffusing population (Fig. 7H,K; diffusive: $16.1 \pm 1.3\%$, mean \pm SEM). However, septin
593 free-diffusion was very slow (Fig. 7J; Fig. S8D) with a diffusion constant $D_{diff} = 0.087 \pm$
594 $0.001 \mu\text{m}^2 \cdot \text{s}^{-1}$ (mean \pm SEM) that is comparable to that of free diffusing transmembrane
595 proteins (integrins: (Rossier et al., 2012)) or of a lipid-anchored protein bound to the
596 plasma membrane by its PH domain (kindlin:(Orre et al., 2021). Confined SEPT9_i3-
597 mEos3.2 was also diffusing very slowly (Fig. S8E) with a diffusion constant $D_{conf} = 0.044$
598 $\pm 0.001 \mu\text{m}^2 \cdot \text{s}^{-1}$ that was comparable to that of mEos2-actin ($0.057 \pm 0.003 \mu\text{m}^2 \cdot \text{s}^{-1}$;
599 means \pm SEM). Overall these results suggest that septins, when immobilized and
600 confined, could indeed be anchoring actin SFs to the plasma membrane, while the free-
601 diffusing septins display a diffusivity that is consistent with them being membrane-
602 anchored.

603 Similarly to the MIET experiments, we used SEPT9_i3-mEos3.2-CAAX as a
604 reference for septins targeted to the plasma membrane. SEPT9_i3-mEos3.2-CAAX did
605 not localize specifically to SFs but decorated the whole plasma membrane (Fig. 7I). In
606 comparison with the behavior of SEPT9_i3-mEos3.2, SEPT9_i3-mEos3.2-CAAX
607 displayed a smaller immobilized fraction (Fig. 7I,J,K; immobile: $32.6 \pm 0.5\%$) but
608 increased free-diffusion and confined diffusion fractions with an increased diffusion
609 constant (D_{diff} : $0.328 \pm 0.002 \mu\text{m}^2 \cdot \text{s}^{-1}$; D_{conf} : $0.202 \pm 0.002 \mu\text{m}^2 \cdot \text{s}^{-1}$, mean \pm SEM) (Fig.
610 S8D,E). Interestingly, being stably anchored to the plasma membrane allowed SEPT9_i3-
611 mEos3.2-CAAX to diffuse inside FAs (Fig. 7I). The much lower diffusion coefficient of
612 SEPT9_i3-mEos3.2 compared to that of SEPT9_i3-mEos3.2-CAAX is in line with freely
613 diffusing SEPT9_i3 being fully incorporated into septin filaments (our results from the split
614 assays) and could reflect hop diffusion of septin filaments alternating between SFs and
615 the plasma membrane. Altogether, our combined findings from SLB assays, MIET and
616 sptPALM in cells support a scenario in which septin filaments in cells function by
617 anchoring SFs to the plasma membrane (working model in Fig. 7L).

618

619 **Microtubule-associated septins organize as filaments containing predominantly**
620 **octamers.** While SF-associated septin filaments contain both SEPT9_i1 and SEPT9_i3-
621 octamers, only octamers containing SEPT9_i1 associate with microtubules (Kuzmic et
622 al., 2022). Co-stainings of septins and MTs in U2OS cells revealed occasionally septin
623 localization to MTs (Fig. 8A), in line with the very low amounts of the SEPT9_i1 isoform
624 in this cell line (Kuzmic et al., 2022). Split-GFP complementation assays probing
625 SEPT9_i1-SEPT9_i1 and SEPT7-SEPT9_i1 interactions, together with mutants
626 disrupting the SEPT9_i1-SEPT9_i1 NC interface and the SEPT9_i1-SEPT7_i1 G
627 interface confirmed that MT localization requires intact SEPT7-SEPT9_i1 and SEPT9_i1-
628 SEPT9_i1 interfaces (Fig. 8B; Fig. S5F-I; Figs. S6Diii,iv and S6E,G). To test whether all
629 septins on MTs also organize as filaments that contain mostly, if not exclusively octamers,
630 we probed SEPT2-SEPT2 interactions on MTs and also selectively removed hexamers
631 using SEPT7Gmut2. To facilitate the study of MT-associated septins in U2OS cells, we
632 increased their amount by co-expressing SEPT9_i1 (Kuzmic et al., 2022; Nagata et al.,
633 2004; Nagata et al., 2003; Surka et al., 2002). Examining the rGFP from SEPT2-SEPT2
634 in live cells, while at the same time imaging MTs, revealed rGFP signal decorating MTs,
635 revealing that MT-associated septins are indeed filamentous (Fig. 8C). The SEPT2 NC
636 interface mutant in the context of the split SEPT2-SEPT2 assay completely abolished MT
637 localization, confirming that an intact SEPT2-SEPT2 NC interface and thus direct SEPT2-
638 SEPT2 interactions are required for septin localization to MTs (Fig. 8D). These results,
639 together with the fact that SEPT2NCmut-mApple fusions do not associate with MTs
640 (Kuzmic et al., 2022), lead us to conclude that all septins decorating MTs are filamentous.

641 Although SEPT9_i1 is essential for septins to bind MTs (Kuzmic et al., 2022),
642 whether hexamers are important for septin filament localization on MTs is not known. In
643 line with our results on exogenously expressed SEPT7, it was difficult to find SEPT9-
644 decorated MTs in cells also displaying ectopic hexamer-based bundles (Fig. S7D,a).
645 rGFP from SEPT7-SEPT7 was, however, readily detected on MTs under our conditions
646 of exogenous SEPT9_i1 co-expression (Fig. 8E), suggesting that the reconstituted split-
647 GFP signal may originate from paired octamers (Fig. 4E). When we expressed
648 SEPT7Gmut2 to selectively disrupt hexamers while preserving octamers, rGFP from
649 SEPT7Gmut2-SEPT7Gmut2 and SEPT7Gmut2-SEPT9_i1 were readily detected on MTs
650 (Fig. 8F; Fig. S6F), reflecting indeed SEPT7-SEPT7 rGFP from paired octamers (Fig. 4E),
651 and also showing that hexamers are not essential for septin-MT association, in line with
652 our findings for actin-associated septin filaments. SIM imaging of MT-associated septin
653 filaments revealed thin septin fibers running along MTs over several micrometers (Fig.
654 8G). Different from the presence of both thin and thick actin-associated septin fibers, all
655 MT-associated septin fibers we observed appeared homogeneous in their width. FWHM
656 measurements of MT and septin fiber widths (Fig. 8H), combined with numerical
657 simulations for estimating the real fiber size in SIM images (Fig. 6G), predicted MT and
658 septin fiber widths in the ranges of 65-135 nm and 85-130 nm, respectively. These

659 predictions are compatible with MT-associated septin filaments organizing as paired
660 septin filaments (Fig. 6I).

661

662 **Discussion**

663 We showed that human septins in U2OS cells extensively associate with myosin-II
664 containing contractile SFs, but not with non-contractile dorsal SFs nor with focal
665 adhesions. Combining a tripartite split-GFP complementation assay to probe SEPT2-
666 SEPT2 interactions as a molecular readout for septin polymerization with mutants that
667 disrupt SEPT2-SEPT2 end-to-end polymerization, we showed that all septins decorating
668 SFs organize as filaments. Moreover, complementation assays probing SEPT9-SEPT9
669 and SEPT7-SEPT9 interactions, together with mutants disrupting the SEPT9-SEPT9 NC
670 interface and the SEPT9-SEPT7 G interface, showed that septin filaments rely on intact
671 octamers. The use of distinct SEPT7 G interface mutants, which either disrupt both the
672 SEPT7-SEPT7 and the SEPT7-SEPT9 G interfaces, or that disrupt specifically the
673 SEPT7-SEPT7 but not the SEPT7-SEPT9 G interface, allowed us to explore the
674 composition of septin filaments and distinguish the contributions of hexamers vs octamers
675 to septin filament integrity and function. Our results showed that septin filaments contain
676 mostly, or even exclusively, octamers, and that octamers, but not hexamers, are essential
677 for the integrity of septin filaments and their function in cell stiffness. Super-resolution
678 structured illumination microscopy revealed that septin filaments organize as fibers
679 running longitudinally along and around SFs, interconnecting SFs and forming a
680 meshwork. Careful analysis of the filament widths in SIM images, using microtubules as
681 a reference, revealed that thin septin fibers most likely correspond to paired septin
682 filaments. To understand the function of septin filaments on SFs, we examined septin
683 localization with respect to the plasma membrane. Nanometer-resolved distance
684 measurements showed that septin filaments associated with ventral SFs are closely
685 apposed to the plasma membrane, in line with reconstitution assays on lipid membranes
686 showing that septins can mediate membrane-actin anchoring and with single protein
687 tracking measurements revealing that septins are largely immobilized and confined on
688 actin fibers. Finally, our results showed that all microtubule-associated septins also
689 organize as filaments whose integrity depend on octamers but not on hexamers.

690 Our study shows for the first time that all SF-associated septins organize as
691 filaments mediated by direct SEPT2-SEPT2 end-to-end polymerization. Whether septins
692 organize invariably as filaments when associated with other actin-based structures in cells
693 and tissues, notably at the cell cortex, at cell-cell junctions and in the cytokinetic ring, can
694 now be tested formally using the tools we have developed in this study, namely a
695 combination of split-GFP assays and polymerization-disrupting mutants. Given the high
696 conservation of residues in the NC interface, our mutants and split assays are well placed
697 for being adapted for other septins within the SEPT2 group when required by cell- and
698 tissue-specific septin expression (Karlsson et al., 2021; Uhlen et al., 2015). Importantly,
699 split assays can test if septins organize as filaments *in situ* in cellular contexts not

700 necessarily linked to actin, and can also be used as readouts for filament formation in the
701 context of future studies aiming to identify regulators of septin polymerization, drugs
702 interfering with septin polymerization, or septin mutants related to disease. These tools
703 can further be adapted for studying septin organization in a tissue context in genetic
704 animal model systems.

705 An unexpected result of our study is the fact that human septin filament integrity in
706 cells depends entirely on octamers, raising the possibility that septin filaments contain
707 predominantly, if not exclusively, octamers, and naturally questioning the functional
708 importance of hexamers. The embryonic lethality of *Sept9* mouse knock-outs (Fuchtbauer
709 et al., 2011) might point to the essential, if not exclusive, contribution of octamers to septin
710 function. Our single point mutant (SEPT7 H279D) that disrupts hexamers while
711 preserving octamers promises to help elucidate the relative functional contribution of
712 hexamers vs octamers in other cellular contexts. Unlike SEPT3 and SEPT12, whose
713 expression is restricted to specific tissues, SEPT9 is expressed in practically all tissues
714 and cell lines (Karlsson et al., 2021; Uhlen et al., 2015), thus octamers are expected to
715 be found ubiquitously. We speculate that it is the SEPT3 group septin, which is absent
716 from hexamers, that dictates septin function; for example, SEPT9_i3 would mediate
717 septin-actin interactions, and SEPT9_i1 would drive septin-microtubule interactions. The
718 fact that recombinant hexamers can also bind actin and membranes (Iv et al., 2021;
719 Szuba et al., 2021) is most likely due to actin- and membrane-binding domains on septins
720 being conserved across septins from different groups and might reflect a fundamental
721 capacity of human septins to interact with these scaffolds; in the presence of the SEPT3
722 group septin, additional physiological signals, for example the very N-terminus of
723 SEPT9_i1 important for septin-MT association (Kuzmic et al., 2022), would work together
724 or in addition to the actin- and membrane-binding domains for determining septin
725 localization and function. It is also possible that the actin/membrane binding of hexamers
726 observed *in vitro* does not happen in cells. The observation that SEPT7 is the septin
727 whose localization and assembly are most sensitive to SEPT7 and SEPT9 expression
728 levels, and the fact that SEPT7-SEPT7 interactions are stronger than SEPT7-SEPT9
729 ones (Rosa et al., 2020) might suggest that SEPT9, and possibly the other SEPT3 group
730 septins, helps prevent SEPT7 from forming ectopic bundles. It is intriguing that *Drosophila*
731 does not have any SEPT3 group septins and thus contains only hexamers (Field et al.,
732 1996). Interestingly, endogenous *Drosophila* septins in cells occasionally form
733 cytoplasmic tube-like bundles devoid of Anillin, in addition to their normal localization to
734 the cortex in interphase cells and to the cleavage furrow in dividing cells (Hickson and
735 O'Farrell, 2008). Such cytoplasmic bundles also form in the absence of Anillin's septin-
736 binding domain that recruits septins to the plasma membrane (Kechad et al., 2012). We
737 speculate that the formation of these cytoplasmic bundles is analogous to the ectopic
738 hexamer bundles we observed in human cells and that they form in similar conditions,
739 i.e., in the absence of, or at limiting amounts of, a physiological partner (SEPT9 in our
740 case, Anillin for *Drosophila*).

741 Our findings suggest that actin-associated septin filaments in mammalian cells
742 organize as paired septin filaments, with thicker septin fibers, e.g. close to FAs, likely
743 consisting of 2-3 double septin filaments. Recombinant and cell-isolated septin protomers
744 in solution and on membranes form single and paired filaments, as well as straight, curved
745 and interconnected bundles of varying thicknesses (DeRose et al., 2020; Iv et al., 2021;
746 Soroor et al., 2021; Szuba et al., 2021). Septins at the budding yeast neck organize in
747 arrays made of single and paired filaments (Bertin et al., 2012; Ong et al., 2014); bundles
748 of more than two filaments were not observed. However, single-molecule localization
749 microscopy of SF-associated septins in U2OS cells, i.e., in the same system we use in
750 our study, reported that septin bundles may comprise as few as 25 to as many as 150
751 filaments (Vissa et al., 2019). However, we note that this study assumed septin GTP-
752 binding domains associating laterally without any spacing, without considering the ~5-20
753 nm spacing occupied by coiled-coil pairing used for bundling (Leonardo et al., 2021), so
754 the number of filaments could be substantially overestimated. The SIM images of SEPT2-
755 GFP in cancer-associated fibroblasts (Calvo et al., 2015) showing thin septin fibers along
756 SFs are in line with our results that septin fibers contain a few rather than tens of filaments.
757 This same study also reported septins appearing to wrap around actin fibers. These
758 observations support the hypothesis that human septins in cells, like budding yeast
759 septins, organize in a rather narrow range of assembly geometries. We speculate that the
760 wide range of assembly geometries found for septins in solution reflects their plasticity,
761 but that the presence of a physiological partner, like actin filaments and the plasma
762 membrane, leads to their native assembly into paired filaments (Bertin et al., 2010; Ong
763 et al., 2014; Szuba et al., 2021), but not to bundles of more than a few filaments, as
764 indicated in our data (Fig. 6H,I).

765 Our results from the MIET assays on ventral SF-associated septins showed that
766 septin filaments are closely apposed to the plasma membrane. We also found septins
767 localized along stretches of peripheral SFs and at curved segments of the membrane that
768 appeared devoid of phalloidin staining (Fig. 6Eiii; Fig. S2B), but these observations are
769 not enough to conclude on direct septin-membrane binding. The strongest evidence, in
770 our opinion, that septins are membrane-bound in cells, or at least closely apposed to the
771 plasma membrane, comes from electron microscopy evidence in budding yeast (Bertin
772 et al., 2012; Ong et al., 2014; Rodal et al., 2005). Our finding that recombinant septin
773 octamer-containing filaments simultaneously bind lipid membranes and actin filaments,
774 thus providing actin-membrane anchoring, leads us to speculate that all SF-associated
775 septins in cells bind actin fibers to the adjacent plasma membrane. Although detectable
776 free-diffusion of septins with sptPALM suggests septin-membrane association, whether
777 septins bind membranes directly or indirectly through membrane-associated interacting
778 partners remains to be shown. It will be interesting to explore if cortical actin meshworks
779 are also membrane-attached via septins.

780 Mammalian septins are distinct and unique from other membrane-bound actin-
781 binding and -crosslinking proteins, like Anillin and ERM proteins, in that they form

782 filaments. Their capacity to form filaments, catalyzed by membrane binding (Szuba et al.,
783 2021) and coupled with their ability to bind and cross-link actin filaments (Iv et al., 2021;
784 Mavrakis et al., 2014), provides them with the unique potential to stabilize actin filament
785 bundles and meshes at the plasma membrane over considerable distances (working
786 model in Fig. 7L). The important fractions of immobilized and confined septins on SFs
787 from sptPALM are in line with such a stabilization function. This hypothesis is further
788 consistent with our SIM imaging showing that septins are intimately associated with actin
789 SFs over many micrometers and also interconnect contractile SFs forming meshes. We
790 propose that septins function precisely by binding ventral SFs, transverse arcs and
791 perinuclear actin caps to the respective ventral and dorsal plasma membrane, stabilizing
792 them at the membrane and at the same time interconnecting the respective SFs,
793 participating in their generation or/and maintenance. Our AFM data are in line with such
794 a role, whereby septins stiffen the cell cortex. Although septins are absent from FAs,
795 multiple reports showed that their absence impacts FA maturation (Calvo et al., 2015;
796 Dolat et al., 2014; Kang et al., 2021). Our observation that septin filaments associate as
797 thick fibers adjacent to FAs lets us think that they contribute a stabilization function at the
798 connection between FAs and the core of SFs and thus impact FA maturation indirectly by
799 affecting the accumulation of mechanical tension on SFs.

800 The findings of this study lead to several remaining open questions. In the absence
801 of the identification of actin-binding domains on septins, it is still unclear if SF decoration
802 by septins in cells reflects direct septin-actin interactions, or if such binding occurs through
803 myosin-II or/and Borg proteins (Calvo et al., 2015; Farrugia et al., 2020; Joberty et al.,
804 2001; Joo et al., 2007; Liu et al., 2014; Mostowy et al., 2010; Salameh et al., 2021). The
805 fact that septins are found only on contractile SFs lets one suppose that myosin-II related
806 signaling might be involved in their recruitment to SFs but this remains to be shown. Also
807 what regulates septin polymerization in cells is still unknown. Cell-free assays have
808 suggested cooperativity in septin-actin binding (Iv et al., 2021), which might drive SF
809 generation and/or maintenance. Cell-free reconstitution approaches and animal model
810 systems promise to provide important further insights into the link between animal septin
811 organization and function.

812
813

814 **Materials and methods**

815

816 **Design of septin fusions for the tripartite split-GFP complementation assay.** For the
817 tripartite complementation assay to report SEPT-SEPT interactions with stringency, the
818 amino acid linker length between SEPT and the β 10- and β 11-strands should not be too
819 short in order to allow for the necessary proximity and flexibility for the β 10- and β 11-
820 strands to orient in an antiparallel fashion for complementing GFP1-9, but it should be
821 short enough to minimize reporting longer-range interactions. We used fluorescence
822 imaging to test the dependence of split-GFP complementation on the linker length and on

823 the position of the β 10- and β 11-tags by screening different homo- and hetero-septin
824 combinations as shown in Fig. S3A-C. All the combinations we tested resulted in
825 fluorescence, reflecting the inherent flexibility of the N- and C-termini of SEPT2, 7 and 9.
826 To allow for the most stringent complementation, we chose to use C-terminal fusions with
827 14-residue linkers for SEPT2- β 10- and - β 11 tags and for SEPT9- β 10- and - β 11 tags, and
828 N-terminal fusions with 14-residue linkers for β 10- and β 11-SEPT7 tags. This short linker
829 is comparable in length to the 10-residue-long β 10- and β 11-strands and thus long
830 enough to allow the antiparallel arrangement of the latter. Protein structure models of
831 human septin hexamers and octamers bearing full-length β 10- and β 11-tagged septins
832 (see method section "Modeling of human septin complexes") confirmed the efficiency of
833 GFP complementation for the final chosen linker length and β 10/ β 11-tag positioning (Fig.
834 2B,C; Fig. 3A,B; Fig. 4D,E).

835
836 **Plasmids and cloning.** Septin and msfGFP cDNAs were as described in (Iv et al., 2021).
837 mApple and sfCherry2 cDNAs were PCR-amplified from Addgene plasmids #54862 and
838 #83031, respectively. Three types of mammalian expression plasmids were used in this
839 study. A pCMV backbone (Clontech) was used for the expression of full-length fluorescent
840 protein (msfGFP/mApple) fusions. A pcDNA3.1 backbone (ThermoFisher Scientific), also
841 with a CMV promoter, was used for the expression of β 10- or β 11-tagged septins. Finally,
842 a pTRIP TRE Bi vector, modified from pTRE-Tight-BI (Takara-Bio) (Koraichi et al., 2018),
843 bearing a bidirectional tetracycline response element (TRE) promoter and an IRES-
844 TagBFP cassette downstream β 10-tagged septins, was used for the doxycycline-
845 inducible co-expression of β 10- and β 11-tagged septins (Fig. S3D,E). pCMV and pTRIP
846 TRE Bi plasmids were used for all results presented in the figures. The pcDNA3.1
847 plasmids were used only for the initial screening (Fig. S3A-C).

848 All pCMV plasmids, SEPT2 constructs in pTRIP TRE Bi plasmids and all interface
849 mutants in pTRIP TRE Bi plasmids were cloned using seamless cloning (In-Fusion HD
850 Cloning Plus Kit from Takara Bio, 638910). All pcDNAs and all wild-type SEPT7 and
851 SEPT9-containing constructs in pTRIP TRE Bi plasmids were generated with classical
852 cloning. In this latter case, DNA fragments were amplified by PCR using the PCR Master
853 Mix from ThermoFisher Scientific (K0171), TaqFast DNA polymerase (Applied Biological
854 Materials G277) or Phusion High-Fidelity DNA Polymerase (New England Biolabs
855 M0530S) and ligated into double digested plasmids with the Rapid DNA Ligation Kit from
856 ThermoFisher Scientific (K1422). pCMV constructs were cloned into a NheI/BamHI
857 linearized vector. pcDNA constructs were cloned into a NheI/XbaI linearized vector.
858 pTRIP TRE Bi constructs were cloned in two steps: first the β 10-tagged septins were
859 cloned into a SacII/NheI digested vector, then the β 11-tagged septins were cloned into a
860 NdeI/XbaI digested vector carrying the β 10-tagged septin. The starting methionine of
861 septin sequences is included in the N-terminal β 10- and β 11-tagged versions.

862 Bacterial expression plasmids for generating wild-type SEPT2-msfGFP hexamers and
863 octamers-9_i3 were described in (Iv et al., 2021) and are available through Addgene

864 (#174492, 174498, 174499, 174501). pNEA-vH plasmids for the bacterial expression of
865 SEPT2NCmut-msfGFP and SEPT2-sfCherry2 were generated using seamless cloning
866 following the same strategy described in (Iv et al., 2021). All primers for seamless cloning
867 were Cloning Oligo (<60 bp) or EXTREmer (>60 bp) synthesis and purification quality
868 from Eurofins Genomics, Germany and are listed in Table S1. All restriction enzymes
869 were FastDigest enzymes from Thermo Scientific or from New England Biolabs. All
870 plasmids were verified by sequencing (Eurofins Genomics, Germany) after each cloning
871 step. We have deposited all plasmids with the nonprofit repository Addgene. Note that
872 the SEPT9_i1NCmut in this study is the same as the SEPT9_i1NCmut2 in (Kuzmic et al.,
873 2022). Plasmid mCherry-SEPT9_i1 was from Addgene (#71622).

874
875 **Cell lines, cell culture and transfection.** U2OS osteosarcoma cells for the expression
876 of full-length fluorescent protein septin fusions were from ATCC (HBT-96). For the
877 inducible co-expression of β 10- and β 11-tagged septins in the context of the tripartite
878 split-GFP complementation system, we generated an inducible U2OS-Tet-On-GFP1-9
879 cell line which expresses constitutively a GFP1-9 fragment and an anti-GFP VHH
880 intrabody that enhances split-GFP fluorescence. To generate this cell line, U2OS cells
881 were successively transduced with lentiviruses encoding rtTA, GFP1-9 and anti-GFP
882 VHH G4 and tested for complementation efficiency using transient expression of a
883 GFP10-zipper-GFP11 domain (Koraichi et al., 2018). One additional round of
884 transduction with GFP1-9 lentivirus lead to an optimized U2OS-Tet-On-GFP1-9 cell line
885 that showed 80% GFP positive cells upon expression of the GFP10-zipper-GFP11
886 domain. An IRES-TagBFP cassette downstream β 10-tagged septins was used for
887 monitoring septin expression. Cells were maintained in McCoy's medium (Gibco
888 16600082) supplemented with 10% fetal bovine serum (Dominique Dutscher S181H), 100
889 U/mL penicillin and 100 μ g/mL streptomycin antibiotics (P4333, Sigma) in a humidified
890 atmosphere at 37°C containing 5% CO₂.

891 Transfections with pcDNAs, for the screening of β 10- and β 11-tag combinations (Fig.
892 S3A-C), were performed 16 h prior to immunostainings using jetPRIME (PolyPlus
893 101000015). To obtain single cells for imaging, 50x10³ U2OS-Tet-On-GFP1-9 cells were
894 typically grown on 18 mm coverslips (Knittel Glass MS0010), previously cleaned by
895 sonication in 70% ethanol, and placed into a 12-well plate a day prior to the day of
896 transfection, for allowing an optimal number of cells to attach and spread. A total of 0.4
897 μ g of DNA and a 4:1 ratio of jetPRIME (μ L) : DNA (μ g) were used per reaction. To
898 minimize septin overexpression artifacts, the total amount of DNA was composed by 30
899 ng of β 10-septin, 30 ng of β 11-septin and 340ng of empty vector.

900 Transfections with either pCMV or pTRIP TRE Bi plasmids and siRNAs were performed
901 through electroporation using the Neon Transfection System (Thermo Fisher Scientific
902 MPK5000). For pCMVs, a single 100- μ L reaction using 1.8x10⁶ U2OS cells, 300 pmol of
903 each siRNA and 6 μ g of each DNA was electroporated within the dedicated tip (Thermo
904 Fisher Scientific, MPK10096). Electroporation parameters consisted in 4 pulses of 10 ms

905 width and a voltage of 1230 V. The electroporated cells were then inoculated in 5 mL of
906 culture medium without antibiotics, and immediately divided for native-PAGE, SDS-
907 PAGE/western blots and immunostaining as follows: 3 mL in a 6-cm dish containing 2 mL
908 of medium without antibiotics, 2 mL in a 6-cm dish containing 3 mL of medium without
909 antibiotics, and 100 μ L in the well of a 12-well plate containing a 18-mm coverslip in 900
910 μ L of medium without antibiotics, respectively. A satisfactory septin knockdown efficiency
911 was achieved within 48-96 h after electroporation. Typically, immunostaining and protein
912 extraction were performed 72h post electroporation.

913 For pTRIP TRE Bi plasmids, a single 100- μ L reaction using 1.8×10^6 U2OS-Tet-On-GFP1-
914 9 cells, 300 pmol of each siRNA and 6 μ g of each DNA was electroporated within the
915 dedicated tip using the same electroporation parameters described previously. The
916 electroporated cells were then inoculated in 5.5 mL of culture medium without antibiotics
917 and immediately divided for SDS-PAGE/western blots, immunostaining and live cell
918 imaging as follows: 5 mL in a 6-cm dish, 400 μ L in the well of a 12-well plate containing
919 a 18-mm coverslip in 600 μ L of medium without antibiotics, and 200 μ L in the well of a
920 24-well glass bottom plate (Cellvis, P24-1.5H-N) containing 800 μ L of medium without
921 antibiotics, respectively. After either 48 h, for samples intended for live cell imaging or
922 immunostainings, or 72 h, for samples for biochemical analysis, protein expression was
923 induced using 1 μ g/mL of doxycycline (Sigma, D9891) for 16 h.

924
925 **Septin mutant phenotype classification.** The diffuse cytosolic vs non-diffuse
926 phenotype classification analysis for mutant characterization with pCMV plasmids (Fig.
927 2I; Fig. S5C,F; Fig. S7A) was done from 3 independent experiments. Transfected cells
928 were fixed and co-stained for actin and α -tubulin. Each round of experiments was
929 composed of the 30 first fluorescent cells found randomly in the sample, with the
930 exception of one round containing 11 cells for msfGFP-SEPT7 and 8 cells for msfGFP-
931 SEPT7Gmut1. Acquired images were classified as "diffuse cytosolic" in the presence of
932 purely diffuse cytosolic signal or as "non-diffuse" in the presence of structure-like signal;
933 no differentiation was applied for SF-, microtubule-, membrane-like or punctate signals in
934 the latter case. The violin graphs representing the phenotype distributions show the mean
935 intensity distribution calculated on the whole field of view from maximum intensity
936 projections of all z-planes. The phenotype classification for split constructs was identical,
937 in terms of the used criteria and graph display, but the data was generated from 2
938 independent experiments, with each experimental round composed of the 20 first
939 fluorescent cells, in live cell imaging, with the exception of one round containing 13 cells
940 for SEPT7Gmut1-SEPT7Gmut1 and 9 cells for SEPT7Gmut2-SEPT7Gmut2. For the
941 septin-actin colocalization analysis, the diffuse cytosolic vs non-diffuse phenotype sorting
942 is displayed both as scatter dot plots and as a pie graph to highlight the diffuse cytosolic
943 vs non-diffuse proportion from each condition. Bars in scatter dot plots depict means and
944 error bars SD. Violin plots, scatter dot plots and pie graphs were prepared using

945 GraphPad Prism. The number of cells used to assess the phenotypes for each condition
946 is indicated in the respective legends.

947

948 **RNA interference.** Control synthetic small interfering RNA (siRNA) targeting the coding
949 region of LacZ (5'-GCGGCUGCCGGAAUUUACC-3') and siRNA targeting the 3'UTR
950 region of all SEPT9 mRNA variants (5'-GGAUCUGAUUGAGGAUAAA-3') were
951 previously validated (Verdier-Pinard et al., 2017). The siRNA sequences targeting the
952 3'UTR regions of SEPT2 and SEPT7 were 5'-ACACUUUCCUGGAUAAAA-3' and 5'-
953 GCAUUUAGCUGUAUUCAUA-3', respectively. All siRNAs were designed to hybridize
954 with 19-bp sequences in the 3'UTR regions of septin genes, thus knocking down
955 endogenous septins while allowing the expression of the transfected plasmids. 21mer
956 siRNAs, 20 nmol each, were synthesized with dTdT overhangs by Eurofins, and delivered
957 as annealed and ready-to-use siRNA duplexes.

958

959 **SDS-PAGE and western blotting of cell lysates.** The dish containing the cells was
960 placed on ice and the cells were washed twice with PBS, without Ca²⁺ and Mg²⁺, before
961 being detached with 40 µL of ice-cold lysis buffer (10 mM Tris-HCl pH 7.5, 148.5 mM
962 NaCl, 0.5 mM EDTA, 0.5% NP-40, 1x PhosSTOP Roche, 5x cOmplete protease inhibitor
963 cocktail Roche, 1 mM DTT) using a cell scraper (TPP 99003). The lysate was collected
964 in a 1.5 mL tube and incubated on ice for 30 min. The lysates were then centrifuged at
965 20,000 g for 20 minutes at 4°C for removing cell debris. An aliquot of 6 µL was collected
966 for protein quantification using the BCA Protein Assay (ThermoFisher Scientific 23227)
967 and the remaining clarified lysates were kept at -20°C until SDS-PAGE analysis.

968 The lysates were analyzed by 4-20% SDS-PAGE using Mini-PROTEAN TGX™ Precast
969 Protein Gels (BioRad 4561095). Molecular mass markers were Precision Plus Protein All
970 Blue Standards (BioRad 1610373) or Amersham ECL Rainbow Marker (Cytiva
971 RPN800E). For the western blot, the gel, the PVDF Immobilon-P^{SQ} membrane (MERCK
972 ISEQ85R), filter pads and filter papers were all incubated in transfer buffer (25 mM Tris,
973 192 mM glycine and 20% of methanol) for 15 min before assembly in the Mini Trans-Blot
974 transfer cell (BioRad 1703935). The transfer was done at 4°C for 16 h at 110 mA constant
975 current. The transfer efficiency was checked by Ponceau S staining (Sigma P7170). The
976 membrane was then blocked in a 3% w/v dry milk TBS-T solution (20 mM Tris-HCl pH7.5,
977 200 mM NaCl and 0.1% v/v Tween20) for 90 min under constant agitation. Primary and
978 secondary antibodies were diluted in the same blocking solution and incubated over the
979 membrane for 90 and 60 min, respectively. In between antibody incubations, membranes
980 were washed three times for 10 min with TBS-T, and the very last wash right before ECL
981 detection was done only with TBS.

982 The loaded amount of extracted protein in the gels was adapted depending on the
983 expression promoter and the analyzed septin. For pCMV plasmids used to assess the
984 knockdown efficiency (Fig. S3F-H), a total of 4 µg of extracted protein was used for
985 detecting endogenous SEPT2, 8 µg for endogenous SEPT7, and 4 µg for endogenous

986 SEPT9. For pTRIP TRE Bi plasmids, a total of 8 μg of extracted protein was used for all
987 analysis. To detect specific septins, we used rabbit anti-SEPT2 (1:2500, Sigma
988 HPA018481), rabbit anti-SEPT7 (1:200, Santa Cruz Biotechnology sc-20620) and rabbit
989 anti-SEPT9 (1:4000, Proteintech 10769-1-AP). For detecting β 10- and β 11-tag
990 expression, we used rabbit anti- β 10 (1:5,000) and rabbit anti- β 11 (1:5,000) (Koraichi et
991 al., 2018). For detecting tubulin as a loading control, we used mouse anti- α -tubulin
992 (1:2,500, Sigma T9026). Secondary HRP-conjugated antibodies were either anti-rabbit-
993 IgG (1:10,000, Cytiva GENA934) or anti-mouse-IgG (1:10,000, Cytiva GENA931).
994 Chemiluminescent detection was performed with an Amersham ImageQuant 800 imager
995 (Cytiva 29399481) using Amersham ECL Select Western Blotting Detection Reagent
996 (Cytiva RPN2235) diluted five times in Milli-Q water. The membrane was incubated with
997 the diluted reagent for 30 s, and washed for 10 s in TBS right before image acquisition.
998 Images were collected in time series mode every 10 s, for a total of 50 images, and
999 processed with ImageQuantTL software for quantification of the band intensities to
1000 measure expression levels. Expression quantification graphs for assessing knockdown
1001 efficiency were prepared using GraphPad Prism and are shown as mean values
1002 (normalized to 1 for siCtrl) with the error bar representing the standard deviation. Data
1003 are from at least 3 independent siRNA treatments.

1004
1005 **Native PAGE and western blotting of cell lysates.** The dish containing the cells was
1006 placed on ice and the cells were washed twice with PBS, without Ca^{2+} and Mg^{2+} , before
1007 being detached with 40 μL of ice-cold native lysis buffer (80 mM PIPES pH 6.9, 2 mM
1008 MgCl_2 , 4 mM EGTA, 0.2% saponin, 5x cOmplete protease inhibitor cocktail Roche). The
1009 lysate was collected in 1.5 mL tube and incubated on ice for 10 min. The lysates were
1010 then centrifuged at 14,000 g for 10 min at 4°C for removing cell debris. To prevent septin
1011 polymerization, clarified lysates were supplemented with NaCl, adding 10 μL of NaCl 5 M
1012 for each 100 μL of lysate. After 15 min of incubation on ice, the lysates were clarified in a
1013 second centrifugation step of 10 min, 14,000 g at 4°C. An aliquot of 12 μL was collected
1014 for protein quantification using the BCA Protein Assay (ThermoFisherScientific 23227),
1015 and the remaining clarified lysates were kept at -20°C until Native PAGE analysis.
1016 The lysates were analyzed by 4-16% Native PAGE using precast Bis-Tris Mini Protein
1017 Protein Gels (Invitrogen BN1003BOX) following the manufacturer's instructions. The
1018 molecular mass marker was NativeMark™ Unstained Protein Standard (Invitrogen,
1019 LC0725). For the western blot, the gel, the PVDF Immobilon- P^{SQ} membrane, filter pads
1020 and filter papers were all incubated in NuPAGE transfer buffer for 15 min before
1021 assembly. The transfer was done at 4°C for 16 h at 20 V constant voltage. The transfer
1022 efficiency was checked by destaining the membrane with an aqueous solution containing
1023 25% of methanol and 10% of acetic acid. The protein marker was identified and the
1024 membrane completely destained with pure methanol for 3 min. The membrane was then
1025 blocked and stained with the respective antibodies as described for SDS-PAGE western
1026 blots.

1027 The loaded amount of extracted protein in the gels was again adapted depending on the
1028 analyzed septin. A total of 10 µg of extracted protein was used for detecting endogenous
1029 or exogenously expressed SEPT2 and SEPT7, and 10 or 4 µg for endogenous or
1030 exogenously expressed SEPT9, respectively. To detect specific septins, we used mouse
1031 anti-SEPT2 (1:7,500, Proteintech 60075-1), rabbit anti-SEPT7 (1:200, Santa Cruz
1032 Biotechnology sc-20620) and rabbit anti-SEPT9 (1:2,000, Proteintech 10769-1-AP).
1033 Secondary HRP-conjugated antibodies were either anti-rabbit IgG (1:10,000, Cytiva
1034 GENA934) or anti-mouse IgG (1:10,000, Cytiva GENA931). Chemiluminescent detection
1035 was done with an Amersham ImageQuant 800 imager (Cytiva 29399481) using
1036 Amersham ECL Select Western Blotting Detection Reagent (Cytiva RPN2235) as
1037 described previously for SDS-PAGE western blot.

1038
1039 **Immunofluorescence.** Cells were fixed for 15 min with 4% paraformaldehyde (Electron
1040 Microscopy Sciences 15714) in 37°C-prewarmed cytoskeleton buffer (10 mM MES pH
1041 6.1, 150 mM NaCl, 5 mM EGTA, 5 mM MgCl₂, 5 mM glucose), followed by 2 x 5 min wash
1042 steps in phosphate-buffered saline (PBS) solution, and a subsequent permeabilization
1043 and blocking step with PBS containing 0.1% saponin and 1% IgG-free/protease free
1044 bovine serum albumin (BSA) (Jackson ImmunoResearch 001-000-161) for 1 h at RT.
1045 Cells were incubated successively with primary antibodies for 16 h at 4°C in a humidified
1046 chamber, followed by secondary Alexa Fluor-conjugated IgG antibodies combined with
1047 0.165 µM Alexa Fluor 647-phalloidin (ThermoFisher Scientific A22287) for 2 h at RT.
1048 Antibody solutions were prepared in PBS containing 0.1% saponin and 1% BSA, and 3 x
1049 10 min wash steps in the same buffer were performed in between antibody incubations.
1050 Coverslips with stained cells were washed 2 x 5 min in PBS and then mounted with 15
1051 µL Fluoromount (Sigma F4680) for image acquisition. Primary antibodies were rabbit anti-
1052 SEPT2 (1:500, Sigma HPA018481), rabbit anti-SEPT7 (1:500, IBL 18991), rabbit anti-
1053 SEPT9 (1:200, Proteintech 10769-1-AP), mouse anti-α-tubulin (1:10,000, Sigma T9026),
1054 mouse anti-paxillin (1:500, Merck Millipore 05-417). Secondary antibodies were donkey
1055 AlexaFluor488-conjugated anti-rabbit IgGs (1:500, Thermo Fisher Scientific A10037) and
1056 donkey AlexaFluor568-conjugated anti-mouse IgGs (1:500, Thermo Fisher Scientific
1057 A21206).

1058
1059 **Immunostaining after live-cell extraction vs after extraction post-fixation.** To live-
1060 extract cells (Fig. 7A), we incubated cells in 37°C-prewarmed cytoskeleton buffer
1061 containing 0.1% v/v Triton X-100 for 1 min, then replaced immediately with 37°C-
1062 prewarmed cytoskeleton buffer containing 4% paraformaldehyde (Alfa Aesar 43368) and
1063 fixed cells for 15 min. Cells were rinsed with PBS and incubated in a
1064 permeabilization/blocking solution of PBS containing 0.1% saponin and 5% goat serum
1065 (ThermoFisher Scientific 16210064) overnight at 4°C. Cells were incubated successively
1066 with primary antibodies for 2 h at RT in a humidified chamber, followed by secondary
1067 Alexa Fluor-conjugated IgG antibodies combined with 0.165 µM Alexa Fluor 546-

1068 phalloidin (ThermoFisher Scientific A22283) for 1 h at RT. Antibody solutions were
1069 prepared in PBS containing 0.1% saponin and 5% goat serum, and 3 x 10 min wash steps
1070 in the same buffer were performed in between antibody incubations. Coverslips with
1071 stained cells were washed 2 x 5 min in PBS and then mounted with Fluoromount-G
1072 (Southern Biotech 0100-01) for image acquisition. Primary antibodies were rabbit anti-
1073 SEPT7 (1:400, IBL 18991) and mouse anti-non-muscle Myosin IIA (1:200, abcam
1074 ab55456). Secondary antibodies were goat AlexaFluor488-conjugated anti-mouse IgGs
1075 (1:400, Thermo Fisher Scientific A11001) and goat AlexaFluor633-conjugated anti-rabbit
1076 IgGs (1:400, Thermo Fisher Scientific A21070). The respective control experiment, i.e.
1077 extracting cells post-fixation (Fig. 7A), involved fixing cells in 37°C-prewarmed
1078 cytoskeleton buffer containing 4% paraformaldehyde for 15 min, then extracting cells with
1079 PBS containing 0.5% Triton X-100 and 5% goat serum for 10 min before overnight
1080 permeabilization/blocking and antibody incubations as described above. This last
1081 protocol was also used for the immunostainings shown in Fig. 1Biv, Fig. 6 and Fig. S2
1082 using the additional primary antibodies mouse anti- α -actinin-1 (1:200, Thermo Scientific
1083 clone BM 75.2) and mouse anti-vinculin (1:200, clone hVin-1 Sigma V9264). Cells shown
1084 in Fig. 1Biv, Fig. 6, Fig. 7A and Fig. S2 were plated on fibronectin-coated coverslips and
1085 left to attach and spread for 6 h before immunostainings. Human plasma fibronectin was
1086 from Millipore (FC010) and was used at 20 μ g/mL in 100 mM bicarbonate buffer pH 8.5
1087 for coating coverslips overnight at 4°C.

1088
1089 **Confocal fluorescence microscopy of cells and image processing.** For live cell
1090 imaging, right before microscopy and due to the absence of CO₂ control on our
1091 microscope setup, the culture medium was exchanged by Leibovitz medium (Gibco
1092 21083027) supplemented with 10% fetal bovine serum and antibiotics. Cells were kept at
1093 37°C in a heating chamber (Okolab H301-T-UNIT-BL). Fluorescence images of live or
1094 fixed cells were acquired using a spinning disk unit (CSU-X1-M1 from Yokogawa)
1095 connected to the side-port of an inverted microscope (Eclipse Ti2-E from Nikon
1096 Instruments) using a Nikon Plan Apo \times 100/1.45 NA oil immersion objective lens, 488-
1097 561- and 641-nm laser lines (Coherent) and an iXon Ultra 888 EMCCD camera
1098 (1024 \times 1024 pixels, 13 \times 13 μ m pixel size, Andor, Oxford Instruments) resulting in an image
1099 pixel size of 65 nm. Z-stacks were acquired with a Δz interval of 0.4 μ m. Exposure times
1100 were in the range of 0.5-3.0 s depending on the exact condition. For the non-diffuse vs
1101 diffuse cytosolic phenotype classification for septin mutant characterization, acquisition
1102 parameters were kept the same among imaging sessions. For actin or microtubule co-
1103 labeling in live cells, cells were incubated for 30 min with 0.5 μ M of SiR-actin or 60 min
1104 with 0.5 μ M of SiR-tubulin and 10 μ M of verapamil in culture medium (SiR Cytoskeleton
1105 Kit, Spirochrome SC006).
1106 Images were processed with the open-source image processing software ImageJ/Fiji. All
1107 shown images, except for the ones used for the septin-actin co-localization analysis that
1108 were acquired as single z-planes, are maximum intensity projections of two consecutive

1109 z-planes contrasted manually in order to optimize the image display. For septin-actin co-
1110 localization measurements, acquired channels of single z-planes, for septin and actin,
1111 were individually processed as follows: images were subjected to automatic contrast
1112 enhancement, allowing 0.1% of saturated pixels, then to a blurring with a Gaussian filter
1113 of radius 1.0 and a subsequent background subtraction using a rolling ball radius of 7
1114 pixels. A manual intensity threshold was used when calculating Pearson and Manders
1115 co-localization coefficients, using the JACoP plugin for ImageJ (Bolte and Cordelières
1116 2006).

1117 Images shown in Fig. 1Biv, Fig. 7A and Fig. S2 were acquired on a Zeiss LSM 710 laser
1118 scanning confocal microscope using a PlanApochromat 100x/1.4 NA oil immersion
1119 objective lens, 488- 543- and 633-nm laser lines for excitation, with all channels at 1AU
1120 for the pinholes. Z-stacks were acquired with a Δz interval of 0.48 μm . All shown images
1121 are single z-planes and were processed with ImageJ/Fiji.

1122

1123 **Super-resolution structured illumination microscopy.**

1124 **Sample preparation and image acquisition.** Cells for super-resolution structured
1125 illumination (SIM) microscopy were plated on high precision ($170 \pm 5 \mu\text{m}$ thick) 18x18mm
1126 glass coverslips from Zeiss (474030-9000-000) and prepared for immunostainings as
1127 detailed in the section "Immunostaining after live-cell extraction vs after extraction post-
1128 fixation"; all images shown in Fig. 6 and Fig. 8G employ extraction post-fixation. For
1129 microtubule stainings used for microtubule width measurements in Fig. 6F,H, cells were
1130 fixed with -20°C -prechilled methanol for 2 min at -20°C and rinsed with PBS before
1131 overnight permeabilization/blocking and antibody incubations as described in the above
1132 section. Primary antibodies were mouse tubulin (1:1,000, Sigma T9026) and rabbit anti-
1133 SEPT7 (1:500, IBL 18991). Secondary antibodies were goat AlexaFluor488-conjugated
1134 anti-mouse IgGs (1:400, Thermo Fisher Scientific A11001) and goat AlexaFluor633-
1135 conjugated anti-rabbit IgGs (1:400, Thermo Fisher Scientific A21070). Images in Fig. 6
1136 were acquired on a Zeiss Elyra PS.1 super-resolution microscope using an alpha
1137 PlanApochromat 100x/1.46 NA DIC M27 Elyra oil immersion objective lens, 488-, 561-,
1138 and 642-nm laser lines for excitation and respective BP495-550, BP570-620 and LP655
1139 emission filters. Z-stacks were acquired with a Δz interval of 0.101 μm . Images were
1140 processed and channel-aligned with the Zeiss ZEN Black software. Images in Fig. 8G
1141 were acquired on a DeltaVision OMX SR (Leica Microsystems/Cytiva) super-resolution
1142 microscope using an Olympus PlanApo N 60x/1.42 NA oil immersion objective lens, 488-
1143 and 640-nm laser lines for excitation and respective 528/48 and 683/40 emission filters.
1144 Z-stacks were acquired with a Δz interval of 0.125 μm . Images were processed and
1145 channel-aligned with the DeltaVision softWoRx 7.0.0 software. All shown images are
1146 single z-planes and were prepared with ImageJ/Fiji.

1147 **Septin fiber diameter and length measurements.** Full width at half maximum (FWHM)
1148 measurements for measuring the diameter of microtubules (MT) and septin fibers in SIM
1149 images (Fig. 6F,H) were made with a custom-generated Matlab code

1150 (FilamentAnalysis.mlx), the source code of which is available at
1151 [gitHub.com/cchandre/Polarimetry](https://github.com/cchandre/Polarimetry). A line was drawn perpendicular to the axis of the MT
1152 or to the long axis of the septin fiber, and the FWHM was extracted from the intensity
1153 profile using the *findpeaks* Matlab function. We measured the width of MTs and septin
1154 fibers at multiple positions along their length and in multiple microtubules and multiple
1155 septin fibers for each SF type per cell. Box plots depicting the distribution of FWHM
1156 measurements (Fig. 6F) were prepared using GraphPad Prism (one data point
1157 corresponds to one width measurement). The central mark indicates the median, and the
1158 bottom and top edges of the box indicate the 25th and 75th percentiles, respectively. The
1159 whiskers extend to the minimum and maximum values. The number of measurements
1160 per condition (MT or SF type) is indicated in the respective legend. A Kruskal-Wallis test
1161 followed by a multiple comparison test was used for comparing the distributions.
1162 Length measurements were implemented in the same custom-generated code. A line
1163 was drawn parallel to the long axis of the septin fiber and the length extracted with the
1164 *curveLength* Matlab function. We measured the length of multiple septin fibers for each
1165 SF type per cell. Scatter dot plots depicting the distribution of length measurements (Fig.
1166 6J) were prepared using GraphPad Prism (one data point corresponds to one length
1167 measurement). The number of measurements per SF type is indicated in the respective
1168 legend. Bars depict median values.

1169
1170 **Numerical simulations for fiber size estimation.** Numerical simulations of the expected
1171 FWHM in SIM images ("image diameter" in Fig. 6G) as a function of the real fiber diameter
1172 ("fiber diameter" in Fig. 6G) were made with a custom-generated Matlab code
1173 (*Convolution_1D.m*), the source code of which is available at
1174 [gitHub.com/cchandre/Polarimetry](https://github.com/cchandre/Polarimetry). A Gaussian point spread function (PSF) was used,
1175 and the curve was generated from the convolution of this PSF with an increasing fiber
1176 diameter size, using the *conv* Matlab function. Assuming a real antibody-decorated MT
1177 diameter size of ~60 nm (Weber et al., 1978), the convolution curve permits to deduce
1178 the PSF size from the measured FWHM in isolated microtubule fibers (the median value
1179 is used). This PSF size being linearly dependent on the emission wavelength, it is then
1180 rescaled to account for the wavelength difference used in MT vs septin imaging: MTs
1181 and septins were imaged at 488 and 642 nm, respectively, for SIM in Fig. 6, whereas MTs
1182 and septins for SIM in Fig. 8G were imaged at 640 and 488 nm, respectively. To predict
1183 the real width of the respective septin fiber diameters (Fig. 6H), the convolution curve was
1184 finally used for the estimated PSF, using as input the measured septin FWHM.

1185
1186 **Production and purification of recombinant human septin complexes.** Wild-type
1187 nonfluorescent and SEPT2-msfGFP hexamers and octamers-9_i3, SEPT2NCmut-
1188 msfGFP hexamers and octamers-9_i3, and SEPT2-sfCherry2 octamers-9_i3 were
1189 produced and purified as described in (lv et al., 2021). Briefly, plasmids expressing
1190 SEPT2, SEPT2-msfGFP or SEPT2NCmut-msfGFP, and plasmids co-expressing SEPT2,

1191 SEPT2-msfGFP or SEPT2NCmut-msfGFP and SEPT6, were co-transformed with
1192 plasmids co-expressing SEPT6 and SEPT7 (Addgene #174499), or SEPT7 and
1193 SEPT9_i3 (Addgene #174501), for generating recombinant nonfluorescent, SEPT2-
1194 msfGFP, or SEPT2NCmut-msfGFP hexamers and octamers-9_i3 (lv et al., 2021).
1195 Plasmids co-expressing SEPT2-sfCherry2 and SEPT6 were co-transformed with
1196 plasmids co-expressing SEPT7 and SEPT9_i3 (Addgene #174501) to generate
1197 recombinant SEPT2-sfCherry2 octamers-9_i3. The N-terminus of SEPT2 is tagged with
1198 a His₆-tag, and the C-terminus of SEPT7 (for isolation of hexamers), or the C-terminus of
1199 SEPT9 (for isolation of octamers), is tagged with a Strep-tag. A purification scheme
1200 comprising a Strep-Tactin affinity column to capture Strep-tagged complexes, followed by
1201 a nickel affinity column to retain the Strep-tagged complexes that also bear His₆-tagged
1202 septins isolates hexamers and octamers (lv et al., 2021).

1203 Co-transformed *E. coli* BL21(DE3) were selected on LB agar plates with carbenicillin and
1204 spectinomycin each at 100µg/mL. A single colony was selected to prepare an overnight
1205 LB medium preculture at 37°C with antibiotics at 100 µg/mL. Terrific broth with antibiotics
1206 at 50 µg/mL, typically 3.5-5 L, was inoculated with the pre-culture and incubated at 37°C.
1207 Bacteria were left to grow to A_{600nm} ~ 0.6-0.8 before inducing expression with 0.5 mM
1208 IPTG for overnight expression at 17°C. The culture was stopped by centrifuging at 3,400
1209 g for 15 min and 4°C, and the supernatants were pooled and further centrifuged at 5,000
1210 g for 10 min and 4°C. Bacteria pellets were stored at -20°C until protein purification.
1211 Bacteria expressing msfGFP- and sfCherry2-tagged septins yield yellow-greenish and
1212 pink-reddish pellets, respectively.

1213 On the day of purification, the pellet was resuspended in ice-cold lysis buffer (50 mM Tris-
1214 HCl pH 8, 300 mM KCl, 5 mM MgCl₂, 0.25 mg/mL lysozyme, 1 mM PMSF, cOmplete™
1215 protease inhibitor cocktail (1 tablet per 50 mL), 10 mg/L DNase I, 20 mM MgSO₄) and
1216 lysed on ice using a tip sonicator with 5 cycles of 30 s "ON", 15 s "OFF". The lysate was
1217 clarified by centrifugation for 30 min at 20,000 g and 4°C, and the supernatant loaded on
1218 a StrepTrap HP column. Strep-tag-II-containing septin complexes were eluted with 50
1219 mM Tris-HCl pH 8, 300 mM KCl, 5 mM MgCl₂, and 2.5 mM desthiobiotin. The pooled
1220 fractions were then loaded to a HisTrap HP column, and His₆-tag-containing complexes
1221 eluted with 50 mM Tris-HCl at pH 8, 300 mM KCl, 5 mM MgCl₂, and 250 mM imidazole.
1222 Only the highest-concentration peak fractions were collected. Both affinity steps were
1223 performed on an ÄKTA pure protein purification system at 4°C (Cytiva). To remove
1224 imidazole, we either performed overnight dialysis or used a PD-10 column, also including
1225 DTT in this last step. The final elution buffer, in which septins are stored, was 50 mM Tris-
1226 HCl pH 8, 300 mM KCl, 5 mM MgCl₂, and 1 mM DTT. Protein concentration was assessed
1227 with absorbance measurements at 280 nm from the calculated extinction coefficients
1228 using ExPASy, and protein aliquots were flash-frozen in liquid nitrogen and stored at -
1229 80°C until further use.

1230 Chemicals used for recombinant septin complex production and purification are as
1231 follows. *E. coli* BL21(DE3) from Agilent (200131). Carbenicillin (C3416), spectinomycin

1232 (S4014), LB broth medium (L3022), LB agar (L2897), SOC medium (S1797) from Sigma.
1233 Terrific Broth from MP Biomedicals (091012017). IPTG (EU0008-C) and lysozyme (5933)
1234 from Euromedex. Imidazole from Fisher Scientific (Fisher Chemical I/0010/53). PMSF
1235 (78830), cOmplete™ Protease Inhibitor Cocktail Tablets (Roche, 11836145001), DNase
1236 I (Roche, 10104159001), *d*-Desthiobiotin (D1411), and DTT (D0632) from Sigma. HisTrap
1237 HP 1 mL columns (17524701) and StrepTrap HP 1 mL columns from Cytiva (28907546).
1238 20K MWCO Slide-A-Lyzer cassettes from Thermo Scientific (87735). PD-10 desalting
1239 columns from Cytiva (17085101).

1240

1241 **Sample preparation for fluorescence microscopy of *in vitro* reconstituted actin and**
1242 **septins.** To prepare flow cells, glass slides and coverslips were cleaned for 15 min in
1243 base-piranha solution (Milli-Q water, 30% ammonium hydroxide, 35% hydrogen peroxide
1244 at a 5:1:1 volume ratio), rinsed with Milli-Q water and stored in 0.1 M KOH up to one
1245 month. Right before assembling flow cells, slides and coverslips were rinsed with Milli-Q
1246 water and dried with synthetic air. Flow cells with ~10 μ L channels were assembled by
1247 sandwiching ~2-mm-wide and ~2.5-cm-long strips of Parafilm between a cleaned glass
1248 slide and coverslip and melting on a hot plate at 120°C. The resulting chambers were
1249 passivated by incubating for 45 min with 1 M KOH, rinsing with actin polymerization buffer
1250 (5 mM Tris-HCl pH 8, 50 mM KCl, 1 mM MgCl₂, 0.2 mM Na₂ATP, 1 mM DTT), incubating
1251 for another 45 min with 0.2 mg/mL PLL-PEG, and rinsing with actin polymerization buffer.
1252 Flow cells were placed in a Petri-dish along with tissue paper soaked in water to prevent
1253 flow channels from drying during the incubation steps and until use.

1254 Lyophilized rabbit skeletal muscle G-actin was resuspended to 5 mg/mL (119 μ M) in G-
1255 buffer (5 mM Tris-HCl pH 8, 0.2 mM Na₂ATP, 0.1 mM CaCl₂, 1 mM DTT), aliquots snap-
1256 frozen in liquid nitrogen and stored at -80°C. Frozen aliquots were thawed and
1257 centrifuged for 30 min at 120,000 g in a benchtop Beckman air-driven ultracentrifuge
1258 (Beckman Coulter Airfuge, 340401) to clear the solution from aggregates. Clarified G-
1259 actin was kept at 4°C and used within 3-4 weeks.

1260 For actin-septin reconstitution experiments, thawed septin aliquots were cleared for 15
1261 min at 120,000 g in a Beckman airfuge right before use. To polymerize G-actin in the
1262 presence of septins, we mixed G-actin, previously diluted with G-buffer to 5 μ M, with
1263 septins, either nonfluorescent ones or msfGFP-labeled septins (at 20% msfGFP molar
1264 ratio for wild-type septins, and 100% GFP for SEPT2NC septins) to a final actin
1265 concentration of 1 μ M and a final septin concentration of 0.3 μ M, right before
1266 polymerization in actin polymerization buffer, additionally containing 1 mM Trolox, 2 mM
1267 protocatechuic acid (PCA), 0.1 μ M protocatechuate 3,4-dioxygenase (PCD) and 0.1%
1268 w/v methylcellulose. To fluorescently label actin filaments, we polymerized G-actin in the
1269 presence of 1 μ M Alexa Fluor 568-conjugated phalloidin.

1270 Actin-septin samples were prepared with a final volume of 10 μ L, were loaded
1271 immediately into passivated flow channels upon mixing of the components to start
1272 polymerization, and flow channels were sealed with VALAP (1:1:1

1273 vasoline:lanoline:paraffin). The contributions of KCl and MgCl₂ from the septin elution
1274 buffer were taken into account to yield the same final composition of actin polymerization
1275 buffer. Actin-septin samples were incubated overnight at room temperature (RT) in the
1276 dark before observation. To polymerize septins in the absence of actin, we followed the
1277 same procedure as above, but replaced the G-actin solution with G-buffer. Septins were
1278 used at 20% msfGFP and 20% sfCherry2 molar ratio for wild-type septins and at 100%
1279 GFP for SEPT2NC septins.

1280 The sources and identifiers for proteins, materials and chemicals are as follows. Glass
1281 slides (26x76 mm) (AA00000102E01FST20) and glass coverslips (24x60 mm)
1282 (BB02400600A113FST0) from Thermo Scientific. Ammonium hydroxide solution
1283 (221228) and hydrogen peroxide solution (95299) from SIGMA. PLL-PEG from SuSoS
1284 AG (PLL(20)-g[3.5]-PEG(2)). Rabbit skeletal muscle G-actin from Cytoskeleton, Inc.
1285 (AKL99). Alexa Fluor 568-phalloidin from Thermo Scientific (A12380). Methylcellulose
1286 (M0512), Trolox (238813), protocatechuic acid (03930590), protocatechuate 3,4-
1287 dioxygenase (P8279) from Sigma.

1288
1289 **Confocal fluorescence microscopy of reconstituted actin-septins and image**
1290 **processing.** Reconstituted actin-septin assemblies were imaged on the same spinning
1291 disk microscope setup described for imaging cells using the same objective lens and
1292 camera. Images were acquired with an exposure time of 0.1 s. Actin-septin bundles were
1293 imaged close to the surface. Septin filament bundles were also found at the surface, but
1294 the clusters of interconnected filament bundles were observed floating in the bulk of the
1295 flow channels. To capture such clusters, z-stacks were acquired over 10-50 μm using a
1296 Δz interval of 0.5 μm. Images were processed with ImageJ/Fiji. Images of actin-septin
1297 bundles are from single planes. Images of septin filament bundles are from maximum-
1298 intensity z projections. The contrast of all images shown was adjusted post-acquisition so
1299 that both dim and bright structures are visible without saturation. All images use an
1300 inverted grayscale, with bright signals appearing black in a white background.

1301
1302 **Metal-induced energy transfer assays**
1303 U2OS cells were transfected with SEPT9_i3-mApple, SEPT9_i3-mApple-CAAX, or
1304 GAP43-mApple with FuGeneHD (Promega E2311). 16h post-transfection, cells were
1305 plated on glass coverslips (for obtaining reference lifetime measurements, see below)
1306 and on gold-coated glass coverslips, previously cleaned with 70% ethanol. Cells were left
1307 to attach and spread for 24h, then fixed for 15 min using 4% paraformaldehyde (Electron
1308 Microscopy Sciences 15714) in cytoskeleton buffer (10 mM MES – pH 6.1 with NaOH,
1309 150 mM NaCl, 5 mM EGTA, 5 mM glucose, 5 mM MgCl₂). The excess of cytosolic protein
1310 content was washed out with a permeabilization/blocking step (0,1% saponin,1% BSA in
1311 PBS) for 1h at room temperature. Labelling of F-actin in U2OS cells was achieved with
1312 AlexaFluor568-phalloidin (Invitrogen A12380) at 165 nM in permeabilization/blocking

1313 solution for 1h. The samples were maintained in PBS until and throughout the
1314 measurements.

1315 Metal-induced energy transfer (MIET) was performed following the concept introduced by
1316 Enderlein and coworkers (Chizhik et al., 2014). Briefly, we measured the fluorescence
1317 lifetime of emitters in the vicinity of a 18 nm-thick gold film. From the calibration of the
1318 fluorescence lifetime dependence with the distance to the gold film (Chizhik et al., 2014),
1319 the distance between the fluorophore and the metal is recovered. The MIET calibration
1320 curve was computed using the MIET-GUI Matlab code developed by the Enderlein group
1321 (https://projects.gwdg.de/projects/miet/repository/raw/MIET_GUI.zip?rev=ZIP). For
1322 mApple, we used a peak emission wavelength at 610 nm and a quantum yield of 49%.
1323 For Alexa Fluor 568, the emission peak was 603 nm and the quantum yield 69%. Our
1324 calculation used the fluorescence lifetime of the dyes measured on a glass coverslip, in
1325 the absence of the metal layer, to account for the slight 0.1 ns lifetime change induced by
1326 the functionalization of the dye to septin, GAP43 or phalloidin. An isotropic orientation of
1327 the fluorophores is assumed (Chizhik et al., 2014).

1328 We used a gold film of 18 nm thickness deposited by electron-beam assisted evaporation
1329 of gold on a borosilicate glass coverslip (Bühler Syrus Pro 710). A 2 nm-thick chromium
1330 layer is used to promote the adhesion of gold on the glass coverslip. For the MIET
1331 calibration, the refractive indexes of the gold and chromium layers were taken from
1332 (Rosenblatt et al., 2020) and (Johnson and Christy, 1974), respectively while the
1333 refractive index of 1.52 for the borosilicate glass coverslip was provided by the supplier
1334 (D 263 M glass by Schott AG).

1335 The fluorescence lifetime measurements were performed with a home built confocal
1336 microscope with a 557 nm iChrome-TVIS laser (Toptica GmbH, pulse duration 3 ps, 40
1337 MHz repetition rate) and a Zeiss C-Apochromat 63x, 1.2 NA water immersion objective.
1338 The excitation power remained below 2 μ W on the sample to avoid photobleaching during
1339 the measurement. The fluorescence light was collected by the same microscope objective
1340 and filtered using a dichroic mirror (ZT 405/488/561/640rpc, Chroma), long-pass filter
1341 (ET570LP, Chroma) and bandpass filter (ET595/50m, Chroma). The confocal pinhole
1342 diameter was 50 μ m. The photon counting detection used an avalanche photodiode
1343 (MPD-5CTC, Picoquant) connected to a time correlated counting module (HydraHarp400,
1344 PicoQuant). The temporal resolution (full width at half maximum of the instrument
1345 response function) was measured to be 38 ps. The fluorescence lifetime histograms were
1346 fitted using SymPhoTime 64 software (PicoQuant GmbH) with a reconvolution taking into
1347 account the measured instrument response function. All the histograms were fitted using
1348 a biexponential function which provided a better fit to the intensity decay than a single
1349 exponential decay. About 20% of the total detected intensity corresponded to the short
1350 lifetime component (below 0.5 ns) which was not considered further for the analysis. The
1351 MIET distance measurements were taken on the long lifetime component which
1352 represented more than 80% of the total detected photons. The distribution of calculated
1353 distances from lifetime measurements for each condition is represented in box plots using

1354 GraphPad Prism (one data point per cell for each condition). The central mark indicates
1355 the median, and the bottom and top edges of the box indicate the 25th and 75th
1356 percentiles, respectively. The whiskers extend to the minimum and maximum values. The
1357 number of cells per condition is indicated in the respective legend. One-way ANOVA
1358 followed by a multiple comparison test was used for comparing the distributions.

1359

1360 **Supported lipid bilayer assays**

1361 **Small unilamellar vesicle formation.** We used three types of lipids, 1,2-dioleoyl-sn-
1362 glycerol-3-phospho-(1'-myo-inositol-4',5'-bisphosphate) (ammonium salt) (PI(4,5)P₂)
1363 (Sigma 850155P), 1,2-dioleoyl-sn-glycerol-3-phosphocholine (DOPC) (Sigma 850375C),
1364 and 1,2-dioleoyl-sn-glycerol-3-phosphoethanolamine-N-(Cyanine 5) (DOPE-Cy5) (Sigma
1365 810335C), all from Avanti Polar Lipids. The lipids were mixed in chloroform, or, in case
1366 PI(4,5)P₂ was present, in a 20:9:1 chloroform:methanol:water mixture in a glass vial. The
1367 organic solvent was then evaporated completely using a stream of N₂ followed by
1368 overnight incubation in a desiccator. The dried lipid film was resuspended in buffer to give
1369 a total lipid concentration of 0.25 mM. We used a sodium citrate buffer of pH 4.8 (50 mM
1370 citrate, made of equal molarity trisodium citrate and citric acid mixed in a 2:3 volume ratio,
1371 50 mM KCl, 0.1 mM ethylenediaminetetraacetic acid) in case PI(4,5)P₂ was present, and
1372 otherwise F-buffer of pH 7.4 (20 mM Tris-HCl, 2 mM MgCl₂, 50 mM KCl, 1 mM DTT). The
1373 lipids were dissolved by four cycles of 1 min vortexing and 5 min incubation. Finally, small
1374 unilamellar vesicles (SUVs) were obtained by sonicating the lipid solution using an
1375 Ultrasonic homogeniser series HD 2000.2 sonicator equipped with a BR30 cup resonator
1376 (Bandelin) at 10% amplitude for 30 minutes with pulses of 5s on and 5s off to avoid
1377 excessive heating.

1378 **Protein preparation.** Unlabelled septin octamers and SEPT2-msfGFP octamers were
1379 purified in house as previously reported (Iv et al., 2021). The protein was stored in aliquots
1380 in septin storage buffer (20 mM Tris HCl pH 7.4, 2 mM MgCl₂, 300 mM KCl, 1 mM DTT),
1381 at -80°C. Before each experiment, unlabelled and labelled septin octamers were mixed
1382 in a 9:1 molar ratio in septin storage buffer at a total concentration of 1800 nM. Lyophilized
1383 monomeric actin (G-actin) from rabbit skeletal muscle (Hypermol 8101-03) was
1384 resuspended following the manufacturer's instructions and dialyzed against G-buffer (5
1385 mM Tris-HCl pH 7.8, 0.1 mM CaCl₂, 0.2 mM ATP, and 1 mM DTT) to remove residual
1386 disaccharides from the freeze-drying process. Protein aggregates were removed by
1387 centrifugation at 148,000 x g for 1h and the supernatant was snap-frozen and stored in
1388 aliquots at -80°C. Fluorescently tagged G-actin was prepared by covalent modification
1389 with Alexa Fluor™ 594 Carboxylic Acid (Thermo Fisher Scientific 15461054) (Alvarado
1390 and Koenderink, 2015). Before experiments, G-actin aliquots were thawed, and any
1391 aggregates were removed by leaving the protein on ice for at least 2h and subsequently
1392 centrifuging at 148,000 x g for 20 min. Unlabelled and fluorescent actin were mixed in a
1393 9:1 molar ratio in G-buffer at a total G-actin concentration of 5 μM.

1394 **Sample preparation.** Supported lipid bilayers (SLB) were formed in custom-made flow
1395 channels made of nr. 1 Menzel coverslips (Thermo Fisher Scientific 11961988) and glass
1396 slides (Thermo Fisher Scientific 11879022). The coverslips and glass slides were first
1397 cleaned in base piranha solution (5% hydrogen peroxide, 5% ammonium hydroxide) at
1398 70°C for 10 minutes, extensively washed with Milli-Q water, and stored in Milli-Q water
1399 for a maximum of 5 days. Just before use, a coverslip and a slide were dried with a stream
1400 of N₂ gas. Flow channels were prepared by sandwiching 2x20 mm parafilm strips
1401 separated by ~3 mm between the glass slide and the coverslip. The parafilm was then
1402 melted by placing the chambers on a hot plate at 120°C and gently pressing on top with
1403 clean tweezers. After cooling down, an SUV solution (7-12 µL, depending on the distance
1404 between the parafilm strips) was pipetted into the channels and incubated in a humid
1405 chamber for at least 20 minutes to promote SUV rupture and SLB formation. Residual
1406 SUVs were removed by washing with 4 channel volumes of F-buffer for DOPC SLBs or
1407 with 2 channel volumes of sodium citrate buffer followed by 2 channel volumes of F-buffer
1408 for 5% PI(4,5)P₂ SLBs. DOPC SLBs contained 99.7% DOPC and 0.3% DOPE-Cy5;
1409 5%PIP₂ SLBs also contained 94.7% DOPC and 0.3% DOPE-Cy5.

1410 Septin octamers and actin were co-polymerized at room temperature in polymerization
1411 buffer (20 mM Tris-HCl pH 7.4, 2 mM MgCl₂, 50 mM KCl, 1 mM DTT, 0.5 mM ATP, 1 mM
1412 GTP) supplemented with 1 mM Trolox to suppress blinking, and an oxygen scavenging
1413 system composed of 1 mM protocatechuic acid and 0.05 µM of procatechuate 3,4-
1414 dioxygenase to minimize photobleaching. We first prepared a 5x master buffer (100 mM
1415 Tris HCl pH 7.4, 10 mM MgCl₂, 5 mM DTT, 2.5 mM ATP, 5 mM GTP, 5 mM Trolox and 5
1416 mM protocatechuic acid). To prepare the sample, we mixed the master buffer (5-fold
1417 dilution), 0.05 µM of procatechuate 3,4-dioxygenase, the G-actin mix (5-fold dilution to
1418 give a final concentration of 1 µM), and the septin mix (6-fold dilution, to give a final
1419 concentration of 300 nM), in that order. The mixture was either immediately added to the
1420 flow channels containing the SLBs and incubated for 1h in a humid environment, or first
1421 incubated in the tube for 1h to promote septin-actin bundle formation and then added to
1422 the flow channels using a cut pipette tip to minimize bundle disruption. The channels were
1423 then sealed with Dow Corning® high-vacuum silicone grease (Sigma Z273554).

1424 **Image acquisition.** The samples were immediately imaged using a Nikon Ti2-E
1425 microscope complemented with a Gataca iLAS2 azimuthal TIRF illumination system. The
1426 sample was illuminated with 488-nm and 561-nm lasers (Gataca laser combiner iLAS2)
1427 to visualize the septin and the actin signals, respectively. The fluorescence signal was
1428 split with a Cairn Research Optosplit II ByPass containing a Chroma ZT 543 rdc dichroic
1429 mirror and filtered with either a 525/50 or a 600/50 chroma bandpass filter. The images
1430 were recorded with an Andor iXon Ultra 897 EM-CCD camera using an exposure time of
1431 50 ms. To check that the SLBs were uniform and free of defects, we examined DOPE-
1432 Cy5 distribution by illuminating with a 642-nm laser filtered with a 708/75 chroma
1433 bandpass filter and recorded using an exposure time of 20 ms. We checked SLB fluidity
1434 by fluorescence recovery after photobleaching of DOPE-Cy5.

1435

1436 **Atomic force microscopy**

1437 **Sample preparation.** 12-mm glass coverslips were coated with 0.1 mg/mL PLL-PEG
1438 (PLL(20)-g[3.5]-PEG(2), Susos) before being illuminated with a deep-UV lamp through a
1439 quartz-chrome photomask bearing the micropattern features (Front Range Photomask)
1440 designed using AutoCAD (Autodesk). We used Y-shaped micropatterns with a spread
1441 area of ~1500 mm². Micropatterned coverslips were then incubated with 25 µg/mL
1442 fibronectin and 5 µg/mL fibrinogen-GFP, the latter for visualizing micropatterns. Wild type
1443 U2OS cells treated with siLacZ siRNA (6mer+8mer), with SEPT9 siRNA (6mer), and
1444 U2OS cells treated with SEPT7 siRNA and also transfected with msfGFP-SEPT7Gmut2
1445 (8mer) were seeded on fibronectin-coated micropatterns 48h post-electroporation. Cells
1446 were incubated for 5-7 h to attach and spread adopting a triangular shape. The expression
1447 of msfGFP-SEPT7Gmut2 for the 8mer condition was confirmed through the detection of
1448 fluorescence in each measured cell.

1449 **Force Spectroscopy experiments and data analysis.** Atomic force microscopy-force
1450 spectroscopy (AFM-FS) was performed on the dorsal perinuclear region of individual cells
1451 at room temperature. We used a MLCT-Bio-DC (D) cantilever featuring a 4-sided regular
1452 pyramid with a semi-open angle of 35°. The spring constant of the cantilevers was
1453 determined in air using the Sader method (Sader et al., 2012) and the optical lever
1454 sensitivity from the thermal spectrum in liquid (Sumbul et al., 2020). Force-distance
1455 curves were acquired applying a maximum force of 0.8 nN with a ramp range of 5 µm, at
1456 the same approach and retract velocity of 5 µm/s on a Nanowizard 4 AFM microscope
1457 (JPK-Bruker). The indentation depth was on the order of 1 µm. 31 cells, 29 cells and 23
1458 cells were probed for the 6mer+8mer, 6mer and 8mer condition, respectively. For each
1459 cell, about 15-30 force curves were acquired across 3 different contact points, resulting
1460 in a total of 576, 630 and 501 force curves for the 6mer+8mer, 6mer and 8mer condition,
1461 respectively. To extract the cell viscoelastic properties, we fitted the Ting numerical
1462 viscoelastic model for a 4-sided regular pyramidal tip of semi-open angle (θ) to the
1463 experimental force-distance curves (Bilodeau, 1992; Efremov et al., 2017):

1464

$$1465 \quad F(t, \delta(t)) = \begin{cases} \frac{3 \tan \theta}{4(1 - \nu^2)} \int_0^t E(t - \tau) \frac{\partial \delta^2}{\partial \tau} d\tau, 0 \leq t \leq t_m \\ \frac{3 \tan \theta}{4(1 - \nu^2)} \int_0^{t_1} E(t - \tau) \frac{\partial \delta^2}{\partial \tau} d\tau, t_m \leq t \leq t_{ind} \end{cases}$$

1466

1467 where F is the applied force; δ is the indentation; t is the time since initial contact, t_m is the
1468 duration of approach trace, t_{ind} is the duration of complete indentation cycle, and t_1
1469 determined by solving the equation

1470

$$1471 \quad \int_{t_1(t)}^t E(t - \tau) \frac{\partial \delta(t)}{\partial \tau} d\tau = 0$$

1472

1473 We assumed that the time-dependent Young's modulus followed a power law
1474 relationship:

1475
$$E(t) = E_0 \left(\frac{t}{t_0} \right)^{-\beta}$$

1476 where E_0 is the elastic modulus at time t_0 , β is the fluidity of the cell and t_0 is the reference
1477 time, arbitrarily assumed 1s. A viscous drag force (F_d) proportional to the trace velocity
1478 (v) was also added to the force traces using a precalibrated value of the viscous drag
1479 coefficient ($b= 5 \text{ pN}\cdot\text{s}/\mu\text{m}$), $F_d=b\cdot v$.

1480 The values of $\log_{10}(E_0)$ and β extracted from each force measurement were pooled by
1481 cell and then averaged. The data was reproduced in 3 independent experiments and their
1482 distribution represented in box plots using GraphPad Prism (one data point per cell for
1483 each condition). The central mark indicates the median, and the bottom and top edges of
1484 the box indicate the 25th and 75th percentiles, respectively. The whiskers extend to the
1485 minimum and maximum values. The number of cells per condition is indicated in the
1486 respective legend. E_0 values (in Pa) were plotted on a log scale. One-way ANOVA
1487 followed by a multiple comparison test was used for comparing the distributions of E_0
1488 values using the $\log_{10}(E_0)$ values, given the log-normal distribution of E_0 .

1489

1490 **Single particle tracking Photo-Activated Localization Microscopy (sptPALM)**

1491 **Cell culture.** Mouse embryonic fibroblasts were cultured in DMEM (Gibco 10313-021)
1492 with 10% fetal calf serum (FCS, Eurobio scientific CVFSVF00-01). Transient transfections
1493 of plasmids were performed 2 days before experiments using the Amaxa nucleofector
1494 (Lonza VPD-1004). The cells were detached with trypsin/EDTA, the trypsin was
1495 inactivated using DMEM with 10% FCS, and the cells were washed and suspended in
1496 serum-free Ringer solution (150 mM NaCl, 5 mM KCl, 2 mM CaCl_2 , 2 mM MgCl_2 , 10 mM
1497 HEPES-Na pH 7.4, 2 g/L glucose), then incubated for 30 min in Ringer solution before
1498 plating on fibronectin-coated glass coverslips (human plasma fibronectin at 10 $\mu\text{g}/\text{ml}$,
1499 Roche 10838039001).

1500 **Plasmids.** SEPT9_i3-mEos3.2 and SEPT9_i3-mEos3.2-CAAX were cloned in a pCMV
1501 plasmid backbone with seamless cloning into a NheI/BamHI linearized vector (primers in
1502 Table S1). EGFP-human β -actin was provided by A. Matus (Friedrich Miescher Institute
1503 for Biomedical Research, Switzerland). The mEos2-actin construct was generated from
1504 EGFP-actin as described in (Rossier et al., 2012). GFP-human paxillin (isoform alpha)
1505 was used as described in (Rossier et al., 2012).

1506 **Optical setup and image acquisition.** sptPALM acquisitions were steered by
1507 MetaMorph software (Molecular Devices) with an inverted motorized microscope (Nikon
1508 Ti) equipped with a temperature control system (The Cube, The Box, Life Imaging
1509 Services), a Nikon CFI Apo TIRF 100x oil, NA 1.49 objective and a Perfect Focus System,
1510 allowing long acquisition in TIRF illumination mode.

1511 Imaging was performed at least 3 hours after seeding the cells on fibronectin-coated
1512 coverslips mounted in a Ludin chamber (Life Imaging Services). For photoactivation
1513 localization microscopy, cells expressing mEos2 and mEos3.2 tagged constructs were
1514 photoactivated using a 405 nm laser (Omicron) and the resulting photoconverted single
1515 molecule fluorescence was excited with a 561 nm laser (Cobolt Jive™). Both lasers
1516 illuminated the sample simultaneously. Their respective power was adjusted to keep the
1517 number of the stochastically activated molecules constant and well separated during the
1518 acquisition. Fluorescence was collected by the combination of a dichroic and emission
1519 filters (D101-R561 and F39-617 respectively, Chroma) and a sensitive EMCCD (electron-
1520 multiplying charge-coupled device, Evolve, Photometric). The acquisition was performed
1521 in streaming mode at 50 Hz. Either GFP-paxillin or GFP-actin were imaged using a
1522 conventional GFP filter cube (ET470/40, T495LPXR, ET525/50, Chroma). Using this filter
1523 cube does not allow spectral separation of the unconverted pool of mEos from the GFP
1524 fluorescent signal. However, with all of the constructs used, whether the mEos signal was
1525 highly or poorly enriched in FAs, we were still able to detect FAs.

1526 **Single molecule segmentation and tracking.** A typical sptPALM experiment leads to a
1527 set of at least 4000 images per cell, analyzed in order to extract molecule localization and
1528 dynamics. Single molecule fluorescent spots were localized and tracked over time using
1529 a combination of wavelet segmentation and simulated annealing algorithms (Izeddin et
1530 al., 2012; Racine et al., 2006; Racine et al., 2007). Under the experimental conditions
1531 described above, the resolution of the system was quantified to 59 nm (Full Width at Half
1532 Maximum, FWHM). This spatial resolution depends on the image signal to noise ratio and
1533 the segmentation algorithm (Cheezum et al., 2001) and was determined using fixed
1534 mEos2 samples. We analyzed 130 2D distributions of single molecule positions belonging
1535 to long trajectories (>50 frames) by bi-dimensional Gaussian fitting, the resolution being
1536 determined as $2.3 s_{xy}$, where s_{xy} is the pointing accuracy.

1537 For the trajectory analysis, FAs ROIs were identified manually from GFP-paxillin or GFP-
1538 actin images. The corresponding binary mask was used to sort single-molecule data
1539 analyses to specific regions. We analyzed trajectories lasting at least 260 ms (≥ 13 points)
1540 with a custom Matlab routine analyzing the mean squared displacement (MSD), which
1541 describes the diffusion properties of a molecule, computed as (Eq. 1):

$$\text{MSD}(t = n \cdot \Delta t) = \frac{\sum_{i=1}^{N-n} (x_{i+n} - x_i)^2 + (y_{i+n} - y_i)^2}{N - n} \quad \text{Eq. 1}$$

1546 where x_i and y_i are the coordinates of the label position at time $i \times \Delta t$. We defined the
1547 measured diffusion coefficient D as the slope of the affine regression line fitted to the $n=1$
1548 to 4 values of the $\text{MSD}(n \times \Delta t)$. The MSD was computed then fitted on a duration equal
1549 to 80% (minimum of 10 points, 200 ms) of the whole stretch by (Eq. 2):

1550

$$\text{MSD}(t) = \frac{4r_{\text{conf}}^2}{3} (1 - e^{-t/\tau}) \quad \text{Eq. 2}$$

1551
1552
1553 where r_{conf} is the measured confinement radius and τ the time constant $\tau = (r_{\text{conf}}^2 / 3D_{\text{conf}})$.
1554 To reduce the inaccuracy of the MSD fit due to downsampling for larger time intervals,
1555 we used a weighted fit. Trajectories were sorted in 3 groups: immobile, confined diffusion
1556 and free diffusion. Immobile trajectories were defined as trajectories with $D < 0.011 \mu\text{m}^2 \cdot \text{s}^{-1}$,
1557 corresponding to molecules which explored an area inferior to the one defined by the
1558 image spatial resolution $\sim (0.05 \mu\text{m})^2$ during the time used to fit the initial slope of the MSD
1559 (Rossier et al., 2012) (4 points, 80 ms): $D_{\text{threshold}} = (0.059 \mu\text{m})^2 / (4 \times 4 \times 0.02 \text{s}) \sim 0.011 \mu\text{m}^2 \cdot \text{s}^{-1}$.
1560 To separate trajectories displaying free diffusion from confined diffusion, we used the time
1561 constant calculated τ for each trajectory. Confined and free diffusion events were defined
1562 as trajectories with a time constant respectively inferior and superior to half the time
1563 interval used to compute the MSD (100 ms). Statistical significance tests were prepared
1564 using GraphPad Prism.

1565
1566 **Modeling of human septin complexes**
1567 Models of full-length human septin complexes were built for analyzing and interpreting
1568 split-GFP experiments. The septin GTP-binding domains (GBDs) used as templates for
1569 the SEPT2, 6 and 7 models using SWISS-MODEL homology modeling software
1570 (Waterhouse et al., 2018) were from PDB 7M6J (Leonardo et al., 2021), the most
1571 complete human septin hexamer structure to date, which includes $\alpha 0$ helices for SEPT6
1572 and 7. As solved in its integrity, the SEPT6 GBD remained unchanged and was used as
1573 is. The SEPT7 GBD structure was completed using SWISS-MODEL. As the use of the
1574 SEPT2 GBD from 7M6J for modeling SEPT2 led to clashes in the modeled SEPT2-
1575 SEPT2 NC interface, the SEPT2 GBD subunit was modeled using the SEPT7 GBD
1576 structure from 7M6J as a template. The lack of structural information for the short N-
1577 terminal extensions of SEPT2, 6, and 7 prompted us to model them as disordered
1578 segments using Phyre2 (Kelley et al., 2015). The C-terminal domains of SEPT2, 6 and 7
1579 were modeled with CCFold (Guzenko and Strelkov, 2018) for the coiled-coil (CC) parts
1580 and Phyre2 for the flexible parts, as detailed in (Iv et al., 2021). The homodimeric parallel
1581 SEPT2CC was used unaltered with respect to (Iv et al., 2021). The previously modeled
1582 SEPT6 and 7 helices in the SEPT6-SEPT7 parallel coiled-coil in (Iv et al., 2021) were
1583 repositioned slightly after comparison with the only parallel septin CC structure to date
1584 (PDB 6WCU) (Leonardo et al., 2021). GBDs, N- and C-terminal extensions were then
1585 combined with PyMOL open-source software. When necessary, the disordered segments
1586 were manually modified to avoid steric clashes and to adjust distances. The SEPT9_i3
1587 model used was the one built for (Iv et al., 2021) and included already N- and C-terminal
1588 extensions. Hexameric SEPT2-SEPT6-SEPT7-SEPT7-SEPT6-SEPT2 and octameric
1589 SEPT2-SEPT6-SEPT7-SEPT9-SEPT9-SEPT7-SEPT6-SEPT2 complexes were built by
1590 fitting the modeled structures to the hexamer from the PDB 7M6J.

1591 To analyze and interpret the split-GFP experiments, the entire constructs used in the
1592 assays, including β 10- and β 11-tagged septins and the reconstituted GFP, were modeled.
1593 To this aim, the split GFP structure (PDB 4KF5) was added to the modeled septin
1594 complexes. The flexible linkers linking the reconstituted GFP to the septin of interest were
1595 built manually using PyMOL; their straight-ish appearance in the models is due to the
1596 polypeptide chains being built as linear structures. To mimic paired septin filaments with
1597 narrow spacing (Leonardo et al., 2021), mediated by homodimeric SEPT2 antiparallel
1598 CCs (Fig. 4E), septin complexes were duplicated and placed parallel to each other with
1599 a gap of ~5 nm. The bent conformation of the septin was built by rotating the CC domain
1600 manually by 90 degrees relative to the GBD. The helices within the homodimeric SEPT2
1601 antiparallel CC were positioned using the antiparallel SEPT4CC structure from PDB
1602 6WB3 as a reference (Leonardo et al., 2021). All manual interventions were realized using
1603 PyMOL.

1604
1605 **Statistics and reproducibility.** The distributions of measurements, or of phenotypes in
1606 the case of septin mutant characterization, are represented with GraphPad Prism using
1607 box plots, violin plots and scatter dot plots as indicated in the respective methods sections
1608 and legends. Bars, error bars (SD or SEM) and box plot features are as indicated in the
1609 respective figure legends. The number of measurements in each plot and the numbers of
1610 experiments are indicated in the respective figure legend or methods. Statistical
1611 significance tests were performed with with GraphPad Prism. The tests applied and the
1612 obtained P values are mentioned in the respective figure legend. Experiments were
1613 repeated at least three times independently to ensure reproducibility. Experiments from
1614 Fig. 7C-F; Fig 7H; Fig. S4 were performed twice. Experiments from Fig. 7G,I were
1615 performed once. No data were excluded from the analyses.

1616
1617 **Data availability.** All data supporting the findings of this study are available within the
1618 article and its supporting information files. The source datasets generated and analyzed
1619 during the current study are available from the corresponding author on reasonable
1620 request.

1621
1622 **Code availability.** The source codes for the custom-generated Matlab codes for
1623 measurements of fiber diameter (FWHM) and length, and for numerical simulations of
1624 expected fiber diameter (FWHM) from SIM images has been deposited to Github. The
1625 respective links are mentioned in the relevant methods sections.

1626
1627
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1659

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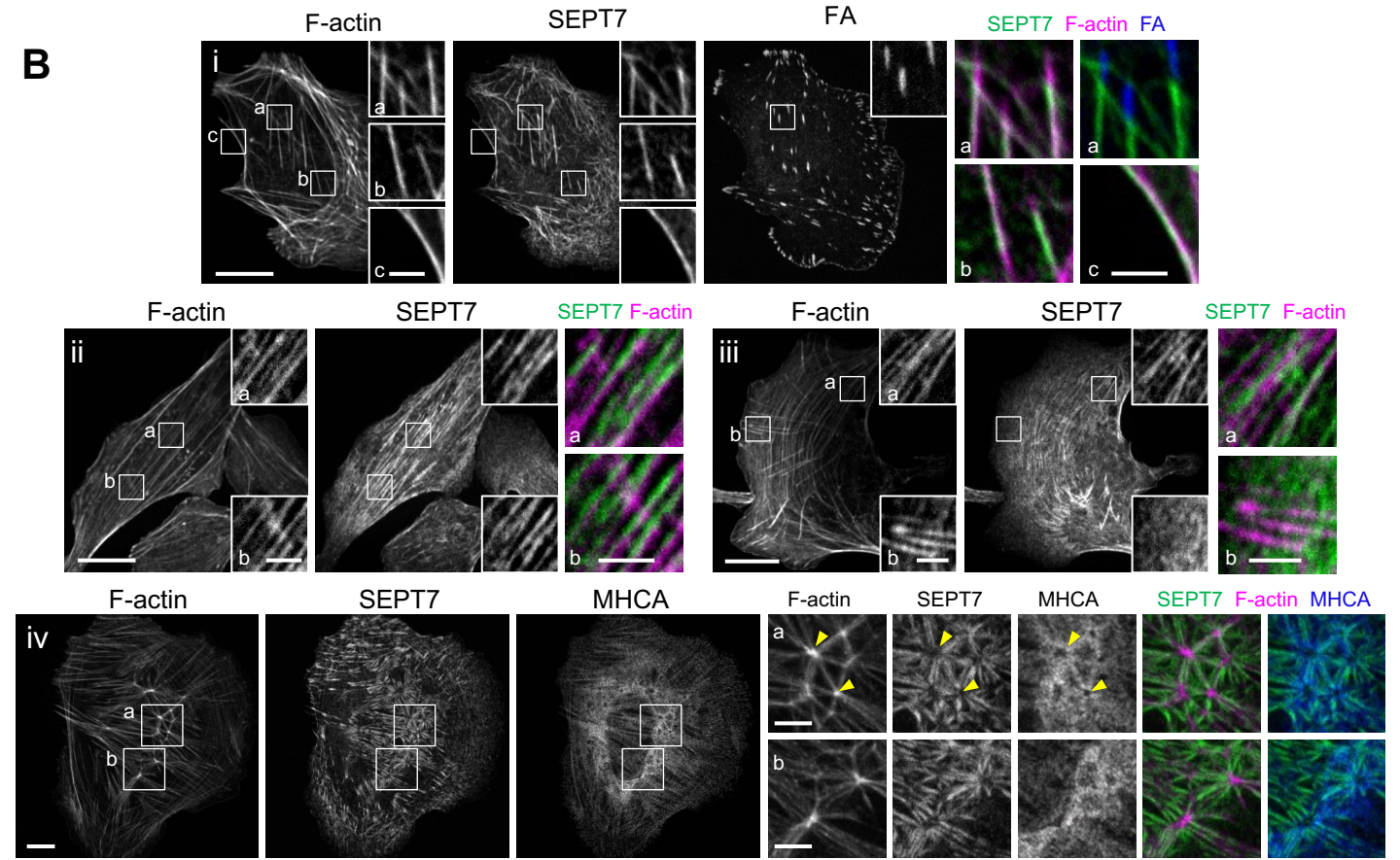
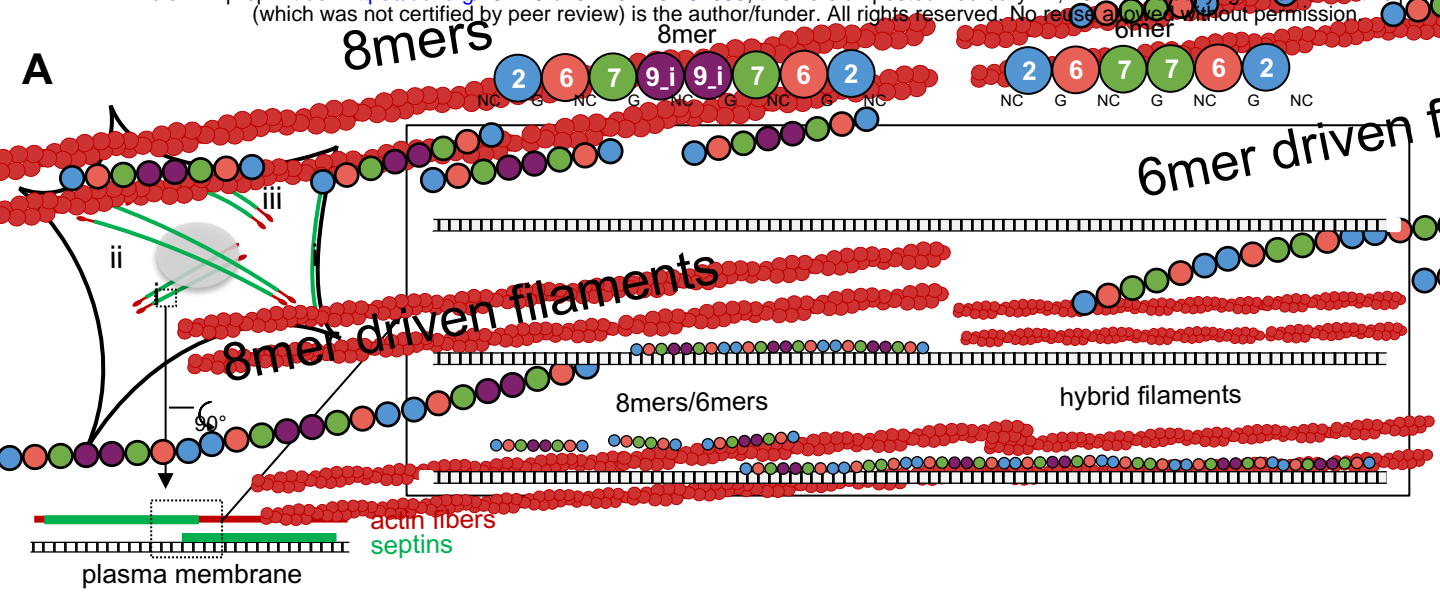
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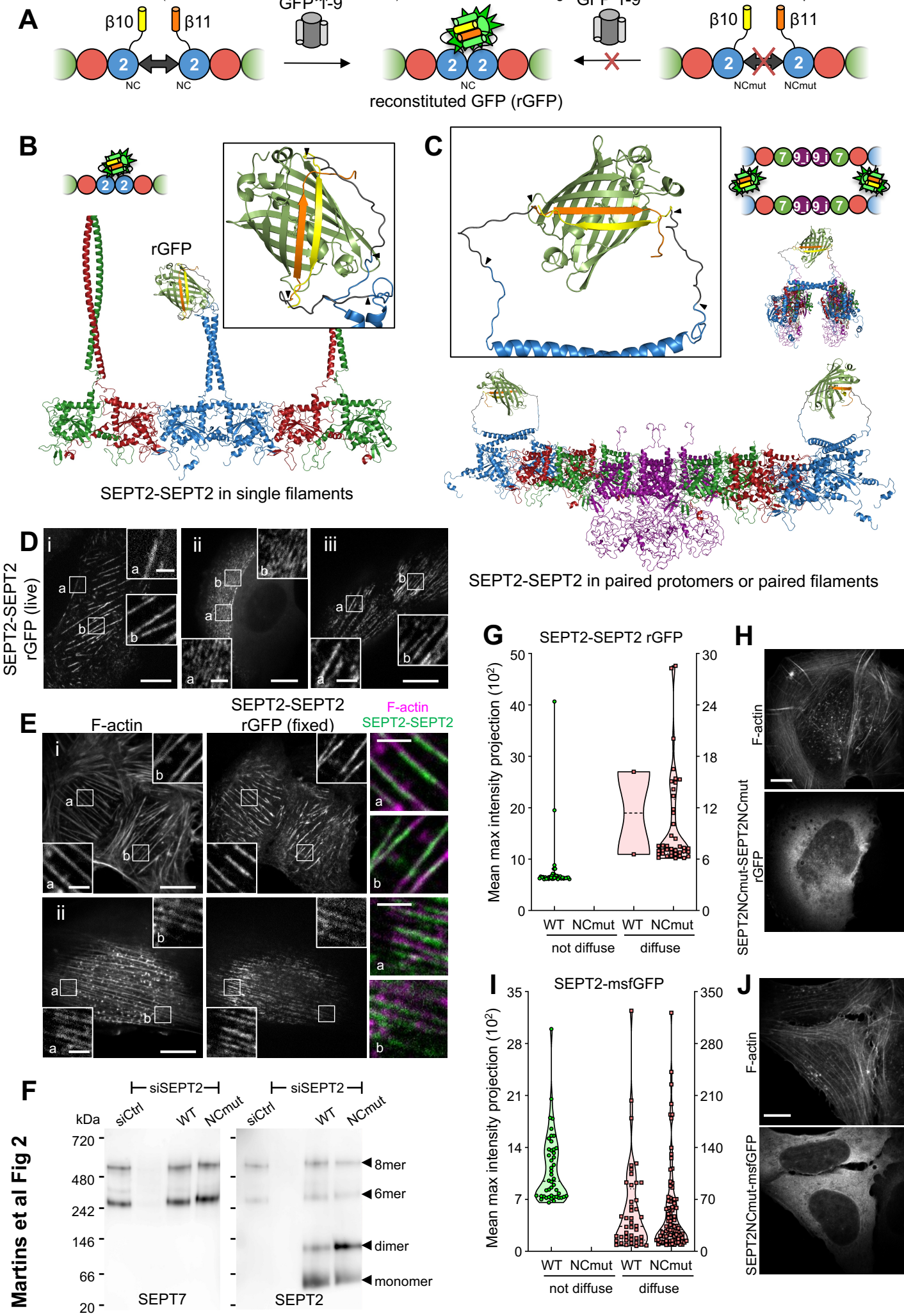
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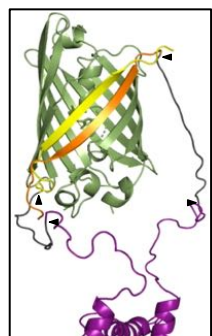
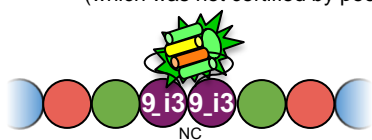


2061 **Figure 1. SEPT7 distribution on different types of stress fibers in U2OS cells. (A)**
2062 The scheme on the left depicts septin-decorated stress fibers (SFs) in a mammalian cell.
2063 Septins (green) decorate different types of actin SFs (red), notably peripheral and ventral
2064 SFs (i), perinuclear actin caps (ii) and transverse arcs (iii), shown in the respective panels
2065 (i-iii) in (B). The cartoons on the right show human septin octamers and hexamers
2066 associating with stress fibers either as single protomers (top), as octamer and hexamer-
2067 driven filaments (middle), or as hybrid filaments from octamer and hexamer co-
2068 polymerization (bottom). Septins can associate exclusively with SFs or also with the
2069 plasma membrane. **(B)** Representative confocal micrographs of SEPT7 immunostainings
2070 showing examples of SEPT7 localizing (i) to ventral (a,b) and peripheral (c) SFs and
2071 excluded from focal adhesions (FA) (a), (ii) to perinuclear actin caps (a,b), (iii) to
2072 transverse arcs (a) and excluded from dorsal SFs (b), and (iv) to ventral actin nodes (a,b).
2073 Cells are co-stained for F-actin (phalloidin), the FA protein paxillin, and myosin heavy
2074 chain (MHCA). Yellow arrowheads in (iv) point to two actin nodes. Scale bars in large
2075 fields of views, 10 μm . Scale bars in insets, 2 μm (i-iii) and 5 μm (iv).



2076 **Figure 2. All septins on SFs organize as filaments. (A)** Schematic representation of
2077 the tripartite split-GFP complementation assay for probing SEPT2-SEPT2 interactions.
2078 The transparency of the SEPT7 subunits is used to suggest that the polymerizing
2079 protomers can be hexamers or/and octamers. **(B-C)** Structure models of reconstituted
2080 GFP (rGFP) via direct SEPT2-SEPT2 interactions of two polymerizing septin protomers
2081 within a filament (B) or from SEPT2 in two apposed protomers (C) (octamers are shown
2082 for simplicity). Only the end-to-end interacting halves of the protomers are shown in (B)
2083 for simplicity; protomers can be hexamers or/and octamers. SEPT6 and SEPT7 coiled-
2084 coils are not shown in (C) for simplicity. The transparency of the terminal SEPT2 subunits
2085 in (C) is used to suggest that the paired protomers could be found within a filament. β 10
2086 and β 11 strands are shown in yellow and orange, respectively. Linker sequences between
2087 septins and the β -strands, delimited by arrowheads, are shown in dark grey. The colors
2088 of septin subunits in the structure models correspond to the ones in the color-coded
2089 sphere representation of hexamers and octamers. The second half of the octamer is not
2090 shown in the rotated filament pair in (C) for the sake of simplicity. **(D-E)** Representative
2091 confocal micrographs of SEPT2-SEPT2 reconstituted GFP (rGFP) distribution in live cells
2092 (D) and in fixed cells (E) co-stained for F-actin (phalloidin). Examples show rGFP in live
2093 cells localizing (i) to peripheral (a) and ventral (b) SFs, (ii) to transverse arcs (a,b), and
2094 (iii) to actin caps (a,b). Examples in fixed cells show rGFP localizing (i) to ventral SFs
2095 (a,b) and (ii) to actin caps (a,b). Scale bars in large fields of views, 10 μ m. Scale bars in
2096 insets, 2 μ m. **(F)** Western blot following native PAGE of U2OS cell lysates probed with
2097 anti-SEPT7 (left) and anti-SEPT2 (right) antibodies upon treatment with siRNAs targeting
2098 LacZ (siCtrl), SEPT2 (siSEPT2), and targeting SEPT2 while expressing wild-type SEPT2-
2099 msfGFP (WT) or SEPT2NCmut-msfGFP (NCmut). Molecular weight markers are shown
2100 on the left. The overexpression of the msfGFP fusions leads to SEPT2 monomers and
2101 dimers in addition to hexamers and octamers (arrowheads). **(G)** Violin plots depicting the
2102 distribution of diffuse cytosolic (red datapoints) vs. non-diffuse (green datapoints)
2103 phenotypes from reconstituted GFP (rGFP) in GFP1-9 cells co-expressing wild-type
2104 SEPT2- β 10 and - β 11 or SEPT2NCmut- β 10 and - β 11. Data points are from a total of 40
2105 cells each for wild-type and mutant SEPT2 distributed among the two phenotypes. **(H)**
2106 Representative example of a GFP1-9 cell co-expressing SEPT2NCmut- β 10 and - β 11
2107 showing a diffuse cytosolic phenotype. Scale bar, 10 μ m. **(I)** Violin plots depicting the
2108 distribution of diffuse cytosolic (red datapoints) vs. non-diffuse (green datapoints)
2109 phenotypes in cells expressing wild-type SEPT2-msfGFP or SEPT2NCmut-msfGFP.
2110 Data points are from a total of 90 cells each for wild-type and mutant SEPT2 distributed
2111 among the two phenotypes. **(J)** Representative example of a cell expressing
2112 SEPT2NCmut-msfGFP showing a diffuse cytosolic phenotype. Scale bar, 10 μ m.

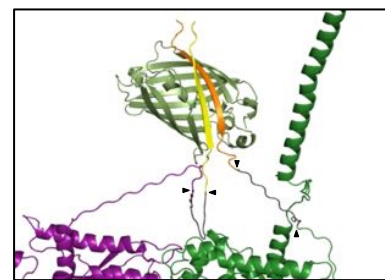
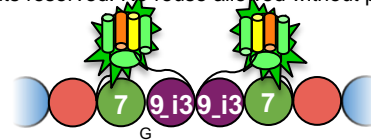
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rGFP

SEPT9_i3-SEPT9_i3

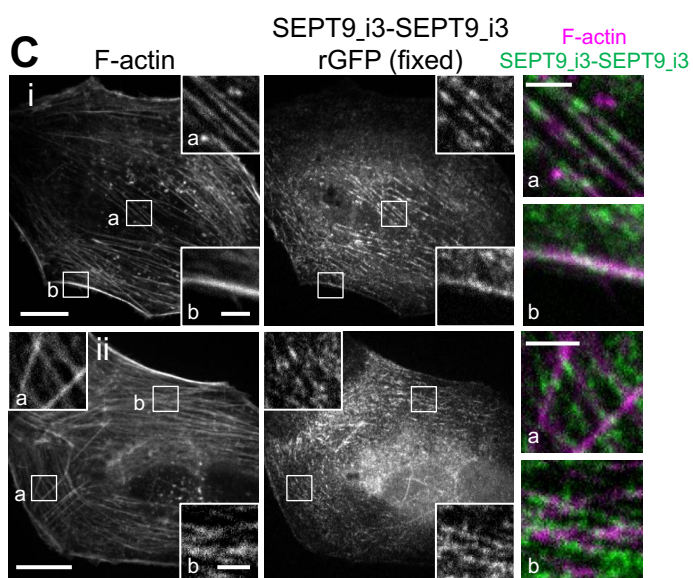
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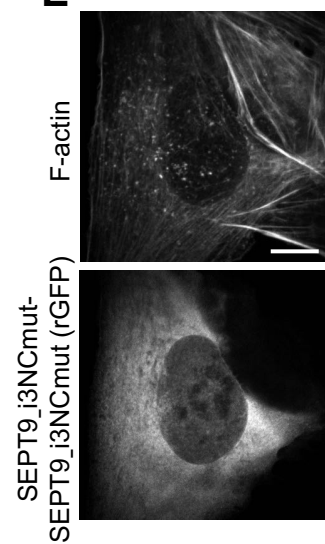
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SEPT7-SEPT9_i3

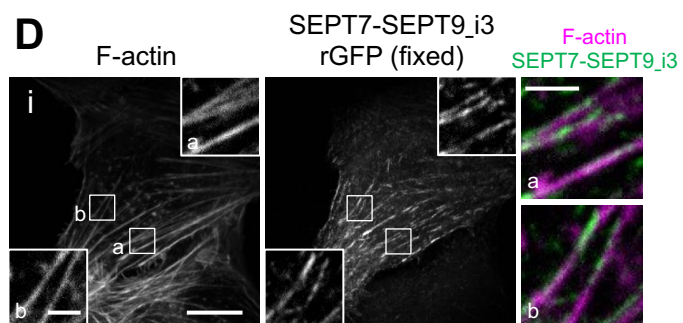
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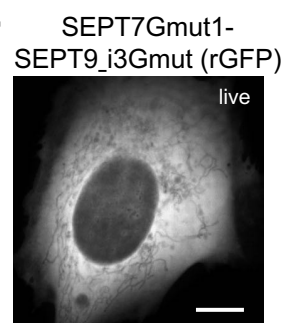
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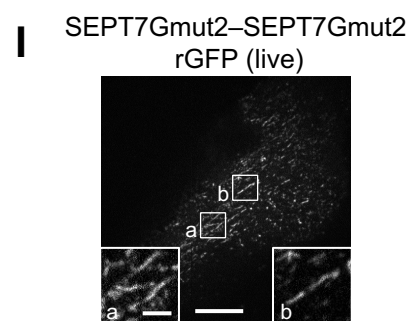
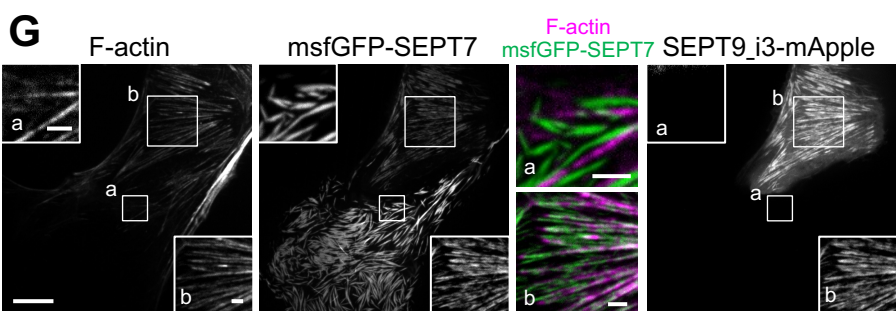
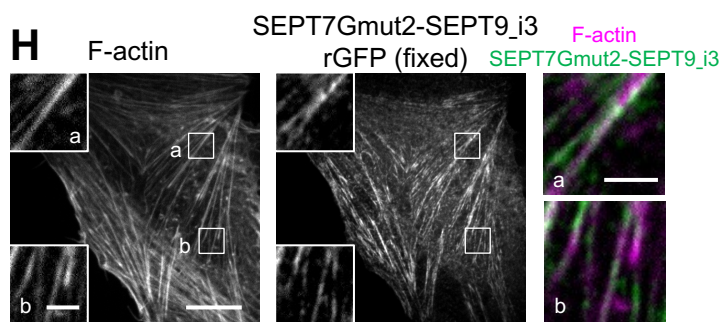
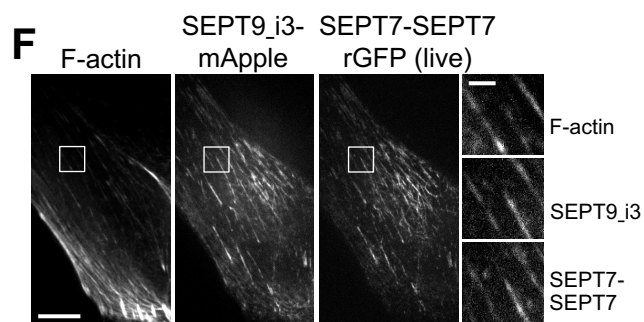
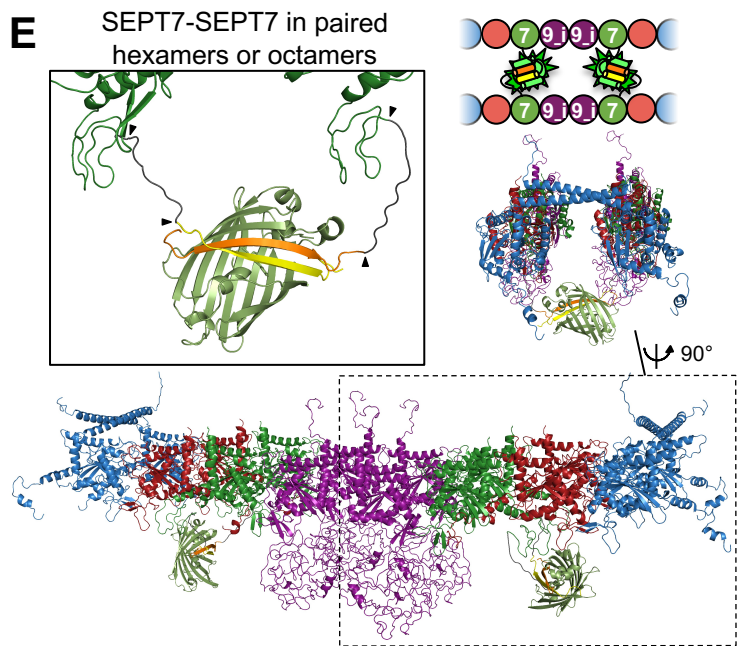
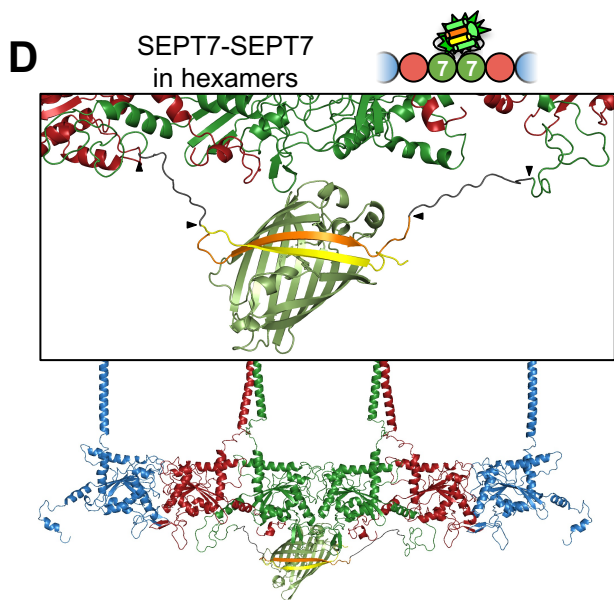
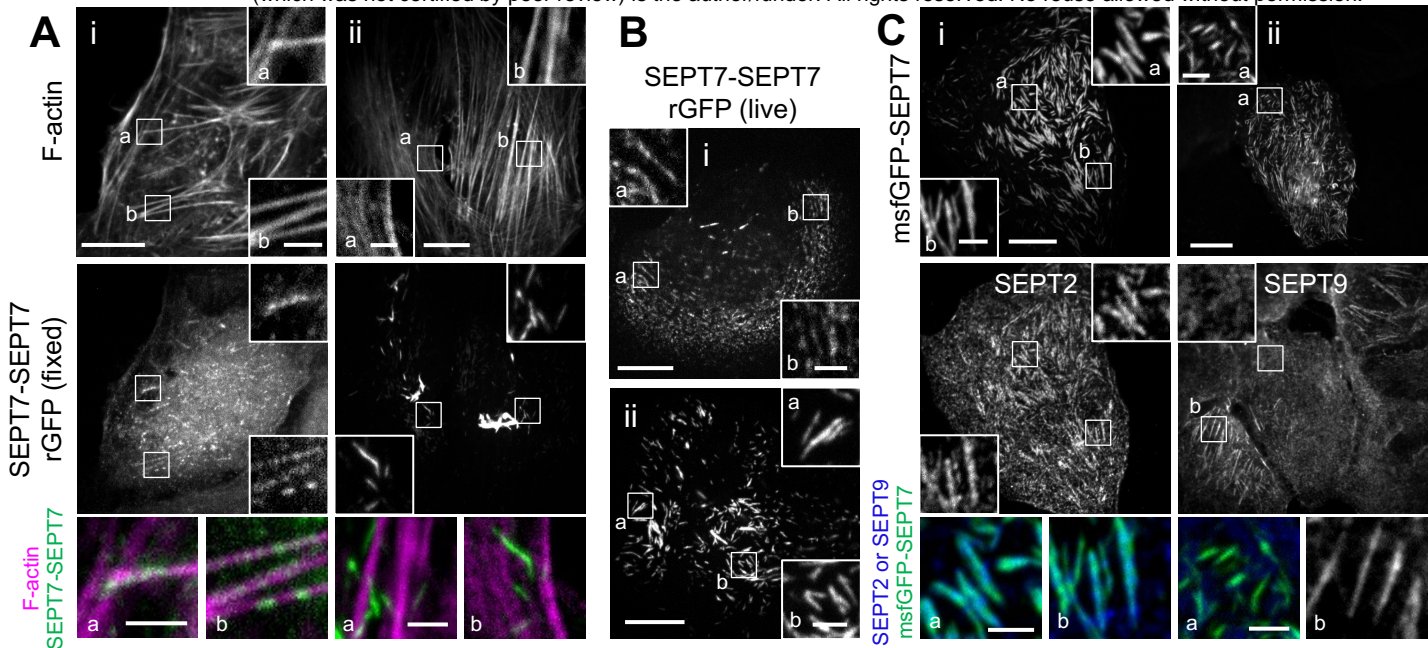
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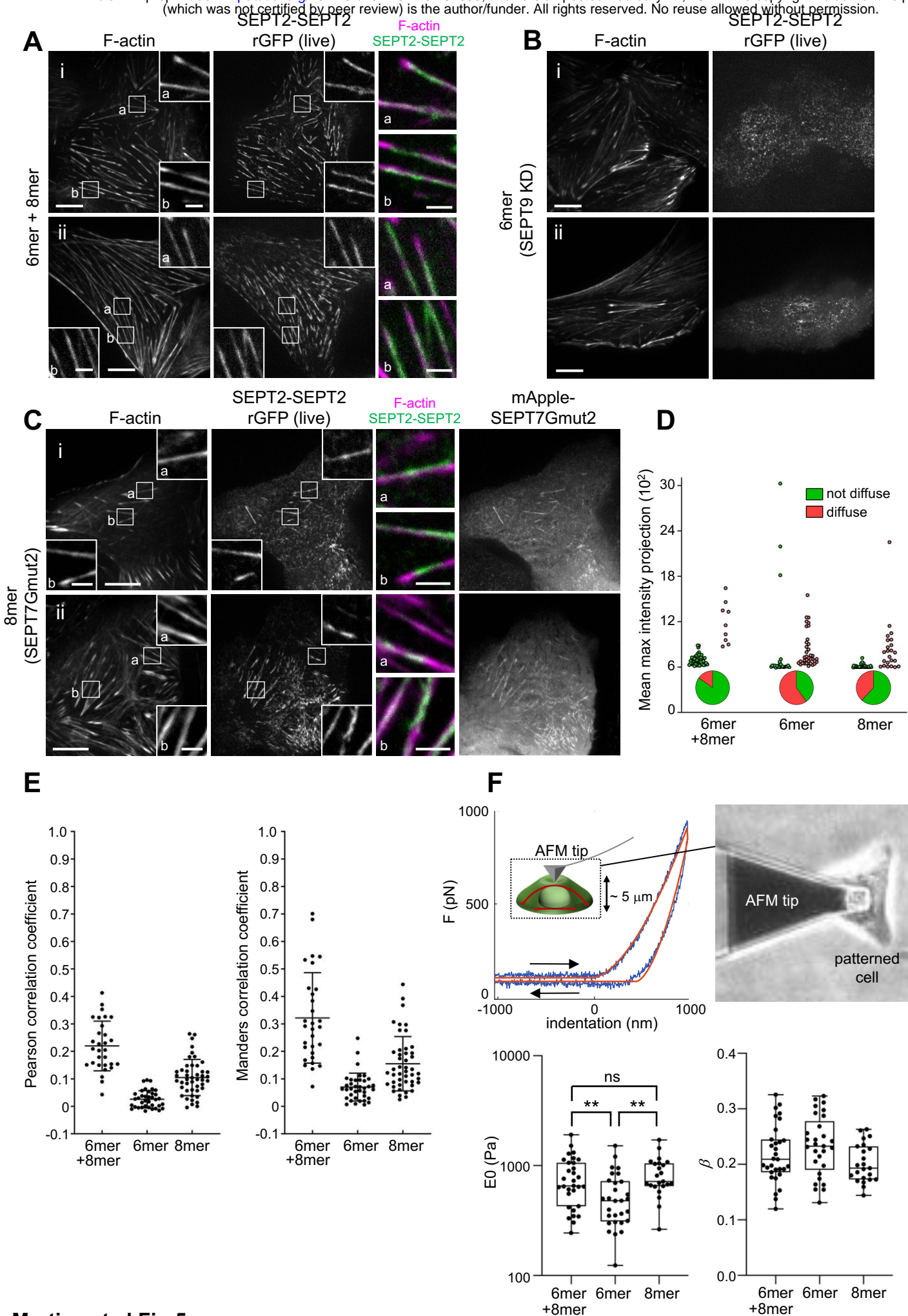
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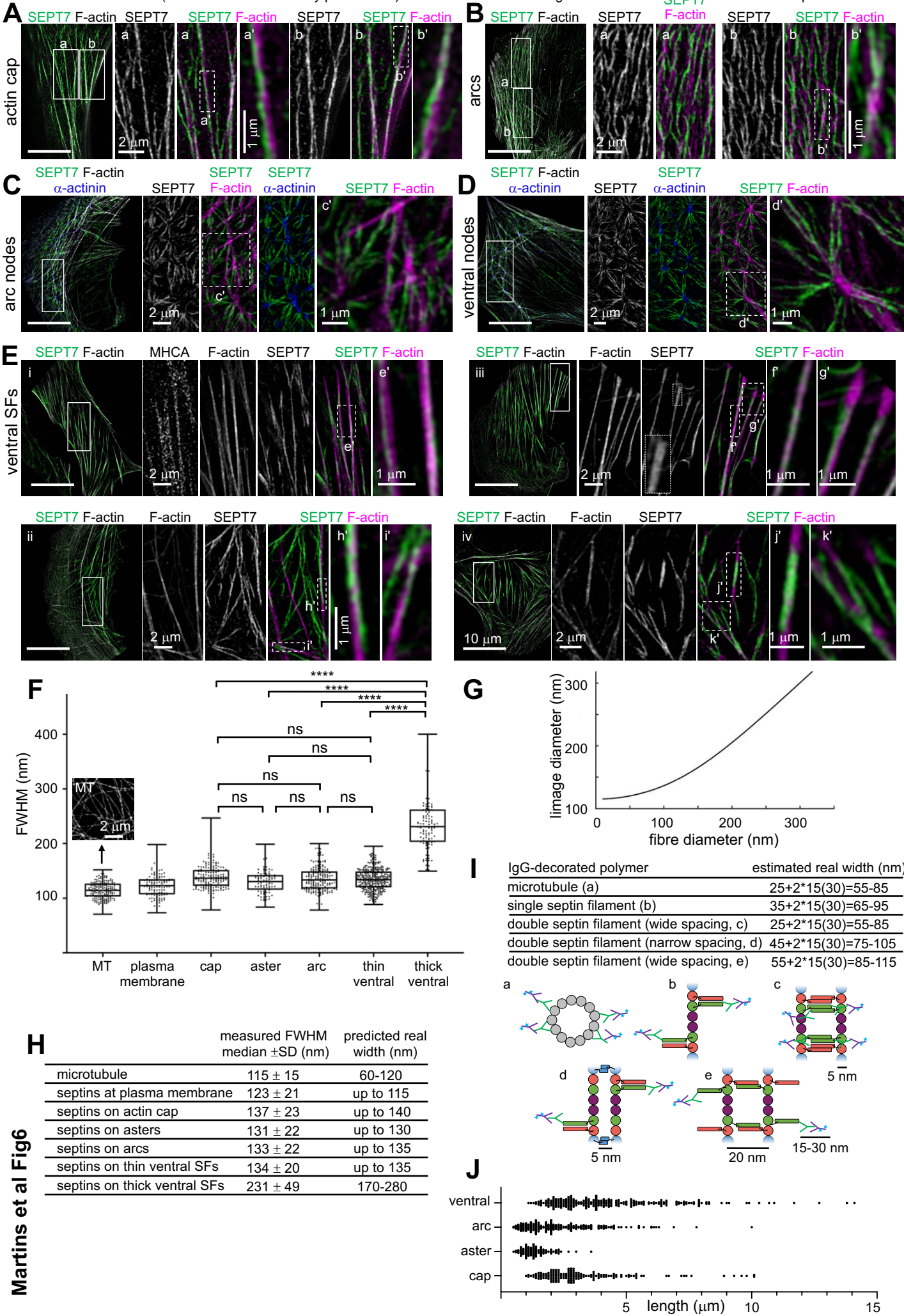
2113 **Figure 3. SF-associated septin filaments contain octamers. (A-B)** Schematic (top) and
2114 respective structure model (bottom) of reconstituted GFP (rGFP) via SEPT9-SEPT9
2115 interactions (A) and SEPT7-SEPT9 interactions (B) within an octamer. The transparency
2116 of the terminal SEPT2 subunits is used to suggest that the protomers are found within a
2117 filament. β 10 and β 11 strands are shown in yellow and orange, respectively. Linker
2118 sequences between septins and the β -strands, delimited by arrowheads, are shown in
2119 dark grey. The colors of septin subunits in the structure models match the ones in the
2120 color-coded sphere representation of octamers. **(C)** Representative confocal micrographs
2121 of SEPT9_i3-SEPT9_i3 rGFP distribution in live cells (right column) and in fixed cells (left
2122 and middle columns) co-stained for F-actin (phalloidin). Examples of rGFP in fixed cells
2123 localizing (i) to ventral (a) and peripheral (b) SFs and (ii) to transverse arcs (b) and
2124 excluded from dorsal SFs (a). Examples in live cells show rGFP localizing (iii) to ventral
2125 SFs (a,b) and (iv) to actin caps (a,b). Scale bars in large fields of views, 10 μ m. Scale
2126 bars in insets, 2 μ m. **(D)** Representative confocal micrographs of SEPT7-SEPT9_i3
2127 reconstituted GFP (rGFP) distribution in live cells (right column) and in fixed cells (left and
2128 middle columns) co-stained for F-actin (phalloidin). Example of rGFP in fixed cells
2129 localizing (i) to ventral SFs (a,b). Example in live cells showing rGFP localizing (ii) to
2130 transverse arcs (a,b). The arrowhead points to a ring (see other examples in Fig. S6C).
2131 Such cytoplasmic rings were \sim 0.5-1.6 μ m in diameter (0.9 μ m on average from 19
2132 measured rings). Scale bars in large fields of views, 10 μ m. Scale bars in insets, 2 μ m.
2133 **(E)** Representative example of a GFP1-9 cell co-expressing SEPT9_i3NCmut- β 10 and -
2134 β 11 showing a diffuse cytosolic phenotype. Scale bar, 10 μ m. **(F)** Representative example
2135 of GFP1-9 cell co-expressing β 11-SEPT7Gmut1 and SEPT9_i3Gmut- β 10 showing a
2136 diffuse cytosolic phenotype. Scale bar, 10 μ m.



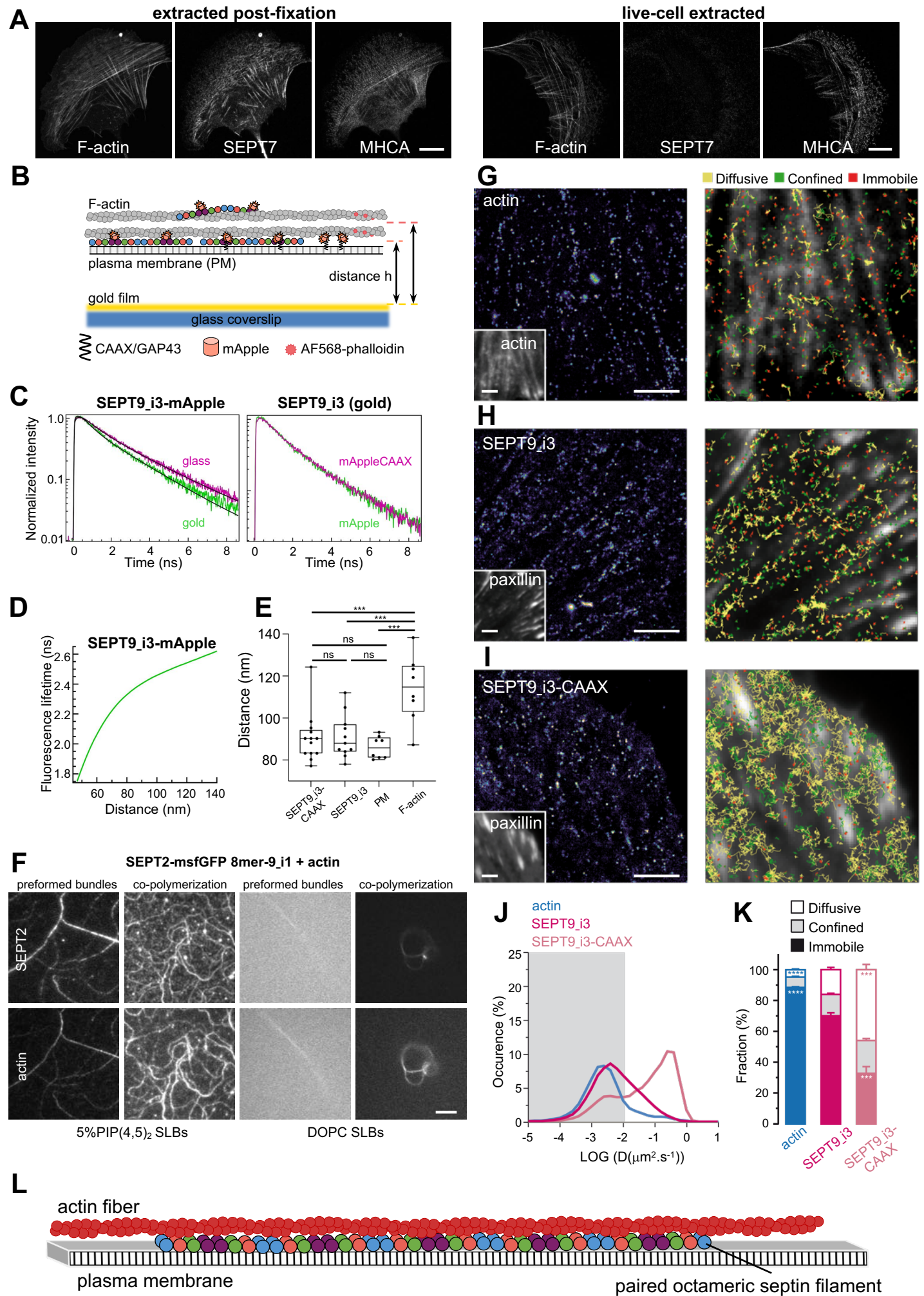
2137 **Figure 4. Exogenous SEPT7 and SEPT9 expression affect SEPT7 distribution. (A)**
2138 Representative confocal micrographs of SEPT7-SEPT7 reconstituted GFP (rGFP)
2139 distribution in fixed cells co-stained for F-actin (phalloidin) localizing (i) to ventral (a,b)
2140 SFs and (ii) to ectopic bundles devoid of phalloidin staining (a,b). Scale bars in large fields
2141 of views, 10 μm . Scale bars in insets, 2 μm . **(B)** Representative confocal micrographs of
2142 SEPT7-SEPT7 reconstituted GFP (rGFP) distribution in live cells localizing (i) to
2143 transverse arcs (a,b) and (ii) to ectopic bundles (a,b). Scale bars in large fields of views,
2144 10 μm . Scale bars in insets, 2 μm . **(C)** Examples of cells expressing msfGFP-SEPT7 and
2145 co-stained for SEPT2 (i) or for SEPT9 (ii). msfGFP-SEPT7 localizing to ectopic bundles
2146 contained SEPT2 (i;a,b) but not SEPT9 (ii;a). A non-transfected cell in (ii) shows SEPT9-
2147 stained SFs (b). Scale bars in large fields of views, 10 μm . Scale bars in insets, 2 μm . **(D-**
2148 **E)** Schematic (top) and respective structure model (bottom) of rGFP via SEPT7-SEPT7
2149 interactions within a hexamer (E) or from SEPT7 in two apposed octamers within a paired
2150 filament (F). The transparency of the terminal SEPT2 subunits is used to suggest that the
2151 protomers are found within a filament. $\beta 10$ and $\beta 11$ strands are shown in yellow and
2152 orange, respectively. Linker sequences between septins and the β -strands, delimited by
2153 arrowheads, are shown in dark grey. The colors of septin subunits correspond to the ones
2154 in the color-coded sphere representation of hexamers and octamers. The second half of
2155 the octamer is not shown in the rotated filament pair in (F) for the sake of simplicity. Only
2156 SEPT7 subunits are shown in the zoom-in of the reconstituted GFP barrel in G for the
2157 sake of simplicity. **(F)** Representative example of a GFP1-9 cell co-expressing $\beta 10$ - and
2158 $\beta 11$ -SEPT7, SEPT9_i3-mApple and labeled for F-actin (SiR-actin). Example shows rGFP
2159 localization to ventral SFs. Scale bars in large fields of views, 10 μm . Scale bars in insets,
2160 2 μm . **(G)** Representative example of a cell (top right) co-expressing msfGFP-SEPT7 and
2161 SEPT9_i3-mApple and labeled for F-actin (SiR-actin). Example shows msfGFP-SEPT7
2162 localizing to ventral SFs (b). A cell expressing only msfGFP-SEPT7 (bottom left) in (ii)
2163 shows msfGFP-SEPT7 localizing to ectopic bundles that are devoid of F-actin (a). Scale
2164 bars in large fields of views, 10 μm . Scale bars in insets, 2 μm . **(H)** Representative
2165 example of a fixed GFP1-9 cell co-expressing $\beta 11$ -SEPT7Gmut2 and SEPT9_i3- $\beta 10$ and
2166 co-stained for F-actin (phalloidin). Example shows rGFP localizing to ventral SFs (a,b).
2167 Scale bars in large fields of views, 10 μm . Scale bars in insets, 2 μm . **(I)** Representative
2168 example of a GFP1-9 cell co-expressing $\beta 10$ - and $\beta 11$ -SEPT7Gmut2 showing rGFP
2169 localizing to SFs. Scale bars in large fields of views, 10 μm . Scale bars in insets, 2 μm .



2170 **Figure 5. Septin octamers are essential for the integrity of SF-associated septin**
2171 **filaments. (A-C)** Representative confocal micrographs of SEPT2-SEPT2 reconstituted
2172 GFP (rGFP) distribution in live cells co-labeled for F-actin (SiR-actin). Cells were treated
2173 with siRNA targeting SEPT2 (A), with siRNAs targeting both SEPT2 and SEPT9 (B), or
2174 with siRNA targeting both SEPT2 and SEPT7 and co-transfected with mApple-
2175 SEPT7Gmut2 (C). Examples in (A) and (C) show rGFP localizing to ventral SFs (a,b).
2176 Scale bars in large fields of views, 10 μm . Scale bars in insets, 2 μm . **(D)** Scatter dot plots
2177 depicting the distribution of diffuse cytosolic (red datapoints) vs. non-diffuse (green
2178 datapoints) phenotypes in cells under the same conditions as in (A-C), also shown as pie
2179 graphs. Data points are from a total of 59 cells for wild-type and 60 cells for each
2180 perturbation condition, distributed among the two phenotypes. **(E)** Scatter dot plots (mean
2181 \pm SD) depicting the distributions of calculated Pearson (left) and Manders (right)
2182 correlation coefficients for actin-septin colocalization in cells under the same conditions
2183 as in (A-C). Data points for each plot, from left to right, are from a total of 30, 37 and 46
2184 cells, respectively. **(F)** Atomic force microscopy nanoindentation on cells under the same
2185 conditions as in A-C. Top, Example of an experimental force-indentation curve. Right and
2186 left arrows correspond to the approach and retraction curves, respectively. The solid red
2187 lines represent the fits. The image on the right shows the cantilever tip indenting the dorsal
2188 membrane of a micropatterned wild-type cell. The cartoon on the left depicts the
2189 indentation of the cell, also showing ventral and dorsal SFs in red. Bottom, box plots
2190 showing the distributions of cell stiffness (E_0) and cell fluidity (β). E_0 values are plotted on
2191 a log scale. The data points are plotted on top of the respective box plots; each data point
2192 corresponds to one cell. On each box, the central mark indicates the median, and the
2193 bottom and top edges of the box indicate the 25th and 75th percentiles, respectively. The
2194 whiskers extend to the minimum and maximum values. The number of measurements in
2195 each box plot, from left to right, is $n = 31, 29, 23$. The respective median cell stiffness
2196 values are 656 Pa, 479 Pa, and 719 Pa, and the respective median cell fluidity values are
2197 0.21, 0.23, and 0.19. One-way ANOVA for $\log(E_0)$; ns=not significant; ** $P < 0.01$.

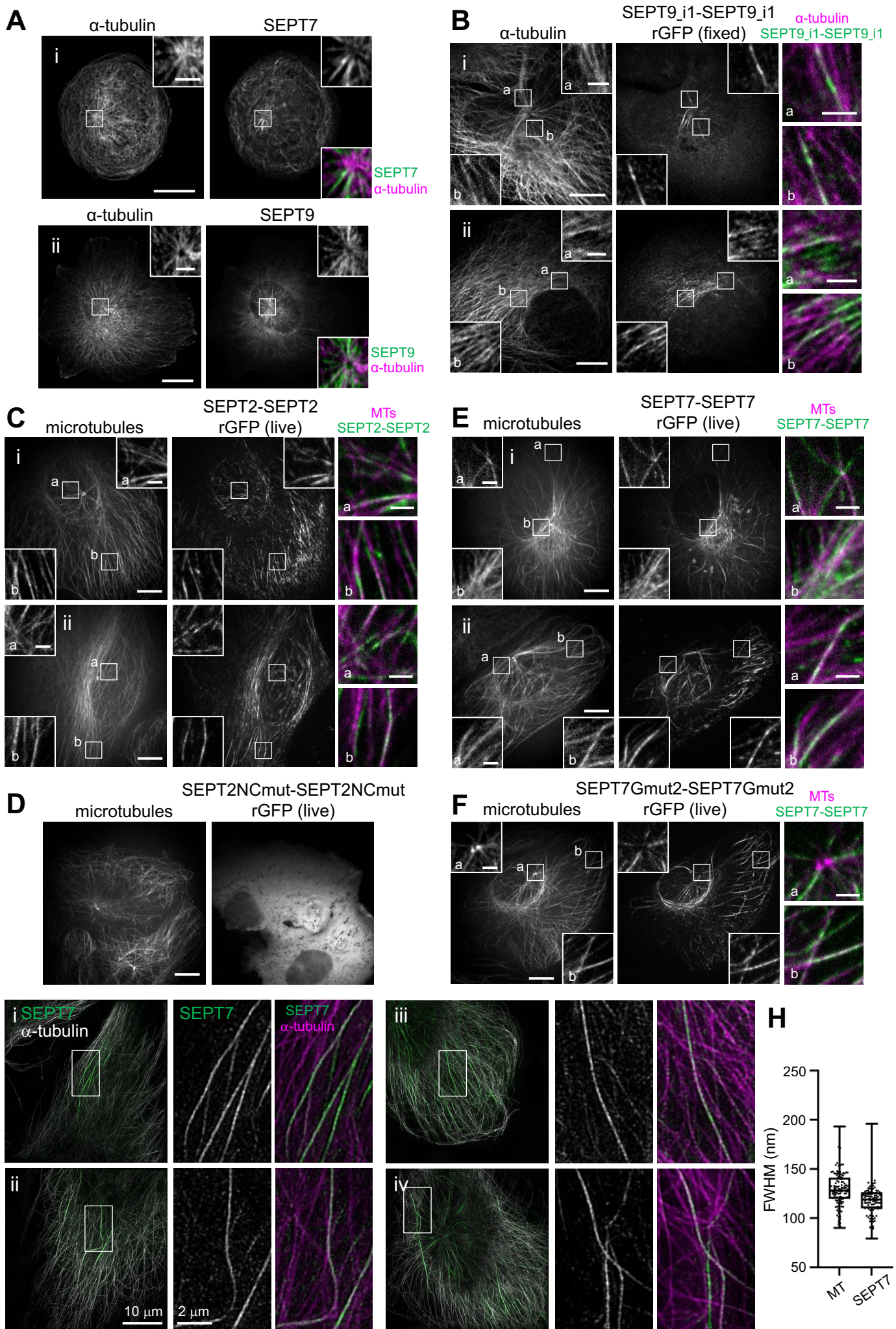


2198 **Figure 6. Super-resolution structured illumination (SIM) microscopy of septin**
2199 **filaments on SFs. (A-D)** Representative SIM micrographs of SEPT7 immunostained
2200 cells co-stained for F-actin (phalloidin) (A-E), and additionally for α -actinin (C,D) or myosin
2201 heavy chain (MHCA) (E). Examples show septin filament localization to perinuclear actin
2202 caps (A), arcs (B) and arc nodes (C), ventral nodes (D), and ventral SFs (E, cells i-iv).
2203 Scale bars in all large fields of views, 10 μm . Scale bars in insets, 1 or 2 μm as indicated.
2204 **(F-I)** Fiber width measurements and real size estimations from SIM images. Box plots in
2205 (F) depict the distributions of measured widths, as the full width at half maximum (FWHM),
2206 of microtubules (MT) (an example of a SIM image of MTs is shown) and septins
2207 associated with peripheral SFs ("plasma membrane"), perinuclear actin caps ("cap"), arc
2208 and ventral actin nodes ("aster"), arcs and ventral SFs; widths from thin and thick septin
2209 fibers were plotted separately. The data points are plotted on top of the respective box
2210 plots; data points correspond to width measurements at multiple positions along MT and
2211 septin fibers and in multiple MT and septin fibers per cell in a total of 10 cells for MT and
2212 10 cells for septin fiber measurements. On each box, the central mark indicates the
2213 median, and the bottom and top edges of the box indicate the 25th and 75th percentiles,
2214 respectively. The whiskers extend to the minimum and maximum values. The number of
2215 measurements in each box plot, from left to right, is $n = 180, 123, 175, 88, 184, 330, 114$.
2216 The respective median values are 115 nm, 123 nm, 137 nm, 131 nm, 133 nm, 134 nm,
2217 and 231 nm. Kruskal-Wallis test; ns=not significant; **** $P < 0.0001$. (G) Numerical
2218 simulations of the expected FWHM in SIM images ("image diameter") as a function of the
2219 real fiber diameter. A Gaussian point spread function (PSF) of 115 nm was used in the
2220 shown graph. The curve was generated from the convolution of the PSF with an
2221 increasing fiber size. Fiber sizes above ~ 200 nm scale linearly with the image sizes.
2222 These simulations were used together with FWHM measurements in SIM images (F) to
2223 estimate an upper width limit for septin fibers associated with the different types of SFs
2224 (H). These estimations were then compared to the real width ranges one expects from
2225 IgG antibody-decorated septins organizing as single or double filaments (I). Primary and
2226 fluorophore (cyan asterisk)-conjugated secondary antibodies are depicted in green and
2227 magenta, respectively. The primary SEPT7 antibody used in our immunostainings binds
2228 the very C-terminus of SEPT7. The narrow and wide spacings of paired filaments, the
2229 presence of homodimeric coiled coils for SEPT2, SEPT6 and SEPT7, and of
2230 heterodimeric coiled coils for SEPT6 and SEPT7 are based on experimental evidence
2231 from (de Almeida Marques et al., 2012; Leonardo et al., 2021; Low and Macara, 2006;
2232 Sala et al., 2016). **(J)** Scatter dot plots of length distributions for septin fibers on the
2233 indicated types of SFs. Bars depict median values. The number of measurements in each
2234 plot, from left to right, is $n = 151, 97, 227, 249$. The respective median values are 2.8 μm ,
2235 1.3 μm , 2.0 μm , and 3.8 μm .

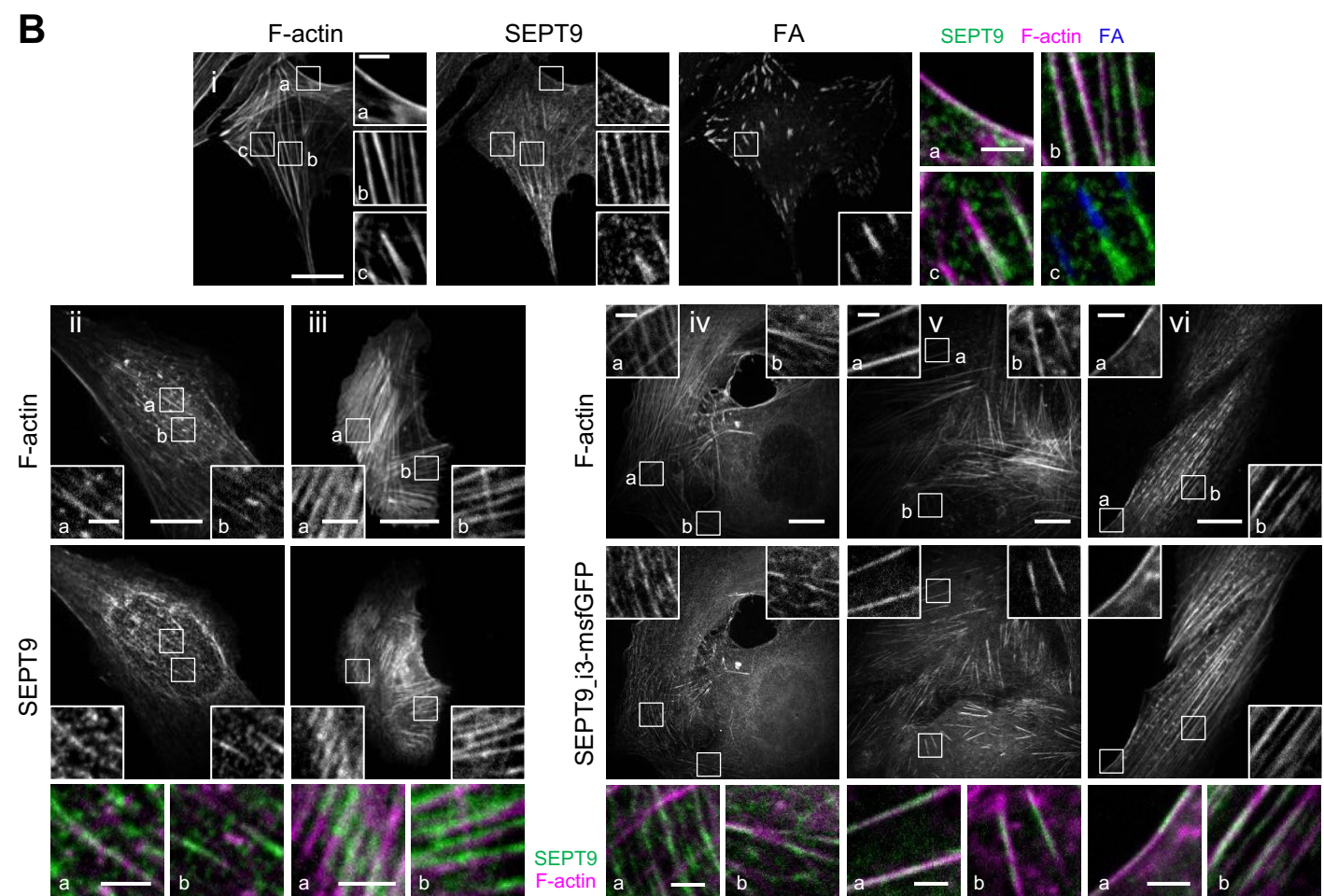
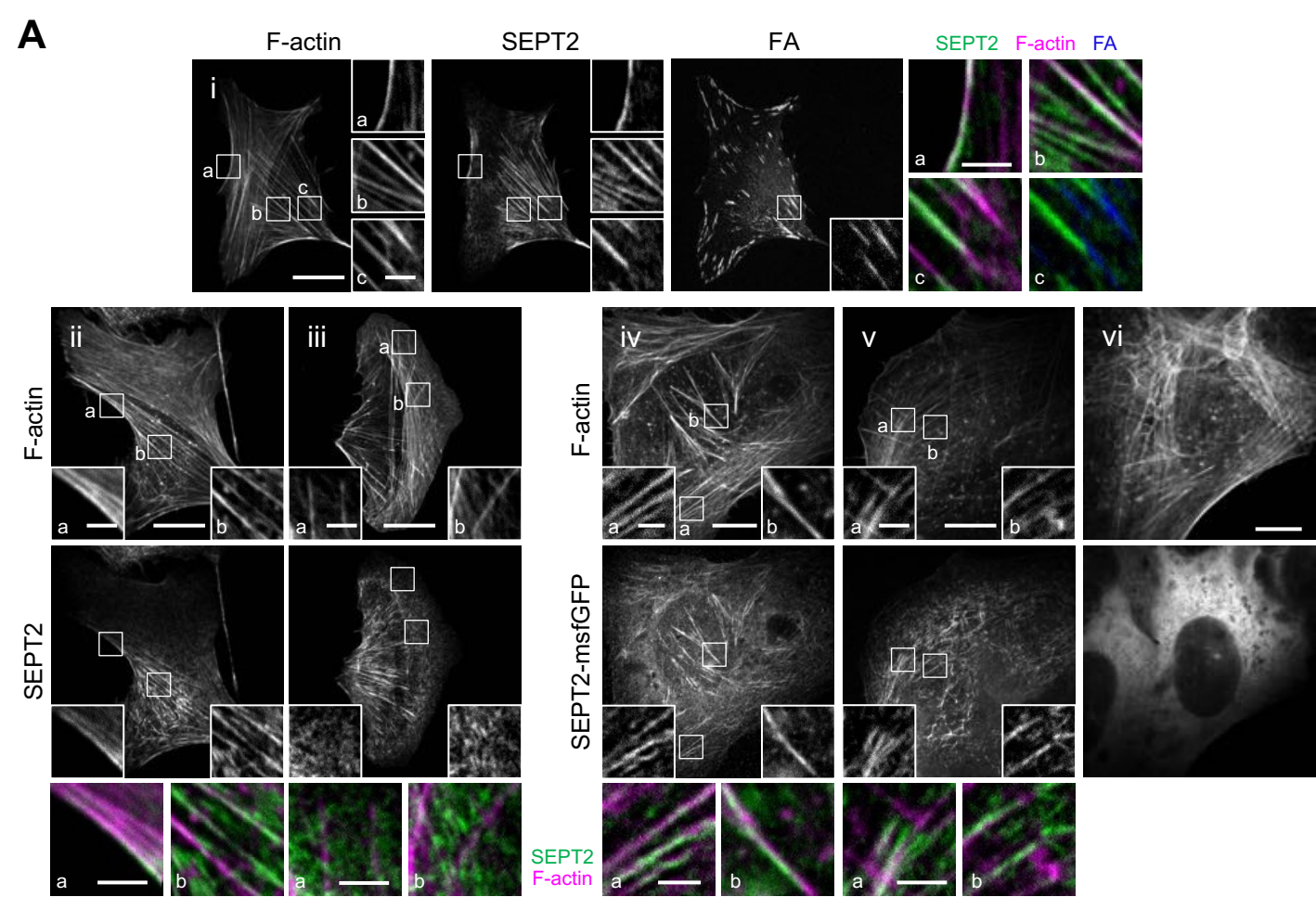


2236 **Figure 7. Septin filaments are closely apposed to the plasma membrane, are largely**
2237 **immobilized on actin stress fibers, and can mediate actin-membrane anchoring. (A)**
2238 Representative confocal micrographs of SEPT7 immunostained cells co-stained for F-
2239 actin and myosin heavy chain (MHCA). Cells were either extracted after fixation (left
2240 panel) or were live-extracted right before fixation (right panel). Scale bars, 10 μ m. **(B)**
2241 Schematic of the metal-induced energy transfer (MIET) assay for probing fluorophore
2242 (mApple or AlexaFluor 568) distances from a gold-coated coverslip using fluorescence
2243 lifetime measurements. **(C-E)** C depicts representative examples of lifetime decay traces
2244 for SEPT9_i3-mApple on glass and in the presence of gold (left) and for SEPT9_i3-
2245 mApple and SEPT9_i3-mApple-CAAX in the presence of gold (right). The solid lines
2246 represent the numerical fits, showing the lifetime reduction due to the MIET process. The
2247 calculated lifetime-distance dependence for SEPT9_i3-mApple (D, see methods) was
2248 used to calculate the distance of SEPT9_i3-fused mApple, with or without the CAAX lipid
2249 anchor, from the coverslip (E). Lifetime decay traces and lifetime-distance dependence
2250 curves for GAP43-mApple (plasma membrane) and AF568-phalloidin (F-actin) are shown
2251 in Fig. S8A,B. Box plots in (E) depict the distributions of calculated distances for
2252 SEPT9_i3-mApple-CAAX, SEPT9_i3, GAP43-mApple (plasma membrane, PM) and
2253 AF568-phalloidin (F-actin). The data points are plotted on top of the respective box plots;
2254 each data point corresponds to one cell. On each box, the central mark indicates the
2255 median, and the bottom and top edges of the box indicate the 25th and 75th percentiles,
2256 respectively. The whiskers extend to the minimum and maximum values. The number of
2257 measurements in each box plot, from left to right, is $n = 13, 11, 8, 8$. The respective
2258 median values are 90 nm, 88 nm, 86 nm, and 115 nm. One-way ANOVA; ns=not
2259 significant; *** $P < 0.001$. **(F)** TIRF images of SEPT2-msfGFP 8mer-9_i1 and F-actin, either
2260 co-polymerized on top of a supported lipid bilayer (SLB), or co-polymerized in solution to
2261 form preformed bundles that were then flushed onto the supported lipid bilayer. The
2262 supported lipid bilayer was composed either of 5% of PI(4,5)P₂, a septin-interacting lipid,
2263 and 95% DOPC (top panels), or 100% DOPC (bottom panels). Due to the shallow
2264 penetration depth (~100 nm) of TIRF together with the absence of crowding agents, only
2265 truly membrane-associated structures are visible. Scale bar, 5 μ m. **(G-K)** Septins are
2266 primarily immobilized and confined in actin stress fibers but also undergo very slow lateral
2267 free diffusion in the vicinity of the plasma membrane. (G-I) Left: Super-resolution PALM
2268 intensity images of mEos2-Actin (G), SEPT9_i3-mEos3.2 (H) and SEPT9_i3-mEos3.2-
2269 CAAX (I) in mouse embryonic fibroblasts obtained from a sptPALM sequence (50 Hz, >80
2270 s). Insets: low resolution images of GFP-actin (G) or GFP-paxillin (H-I), which were co-
2271 expressed for FA labelling. Scale bars, 3 μ m. Right: color-coded trajectories overlaid on
2272 FAs labelled by GFP-paxillin or on FAs and SFs labelled by GFP-actin (grayscale) show
2273 the diffusion modes: free diffusion (yellow), confined diffusion (green) and immobilization
2274 (red). (J) Distributions of the diffusion coefficient D computed from the trajectories of
2275 mEos2-actin (blue), SEPT9_i3-mEos3.2 (magenta) and SEPT9_i3-CAAX-mEos3.2 (light
2276 magenta) obtained outside FAs, are shown in a logarithmic scale. The gray area including

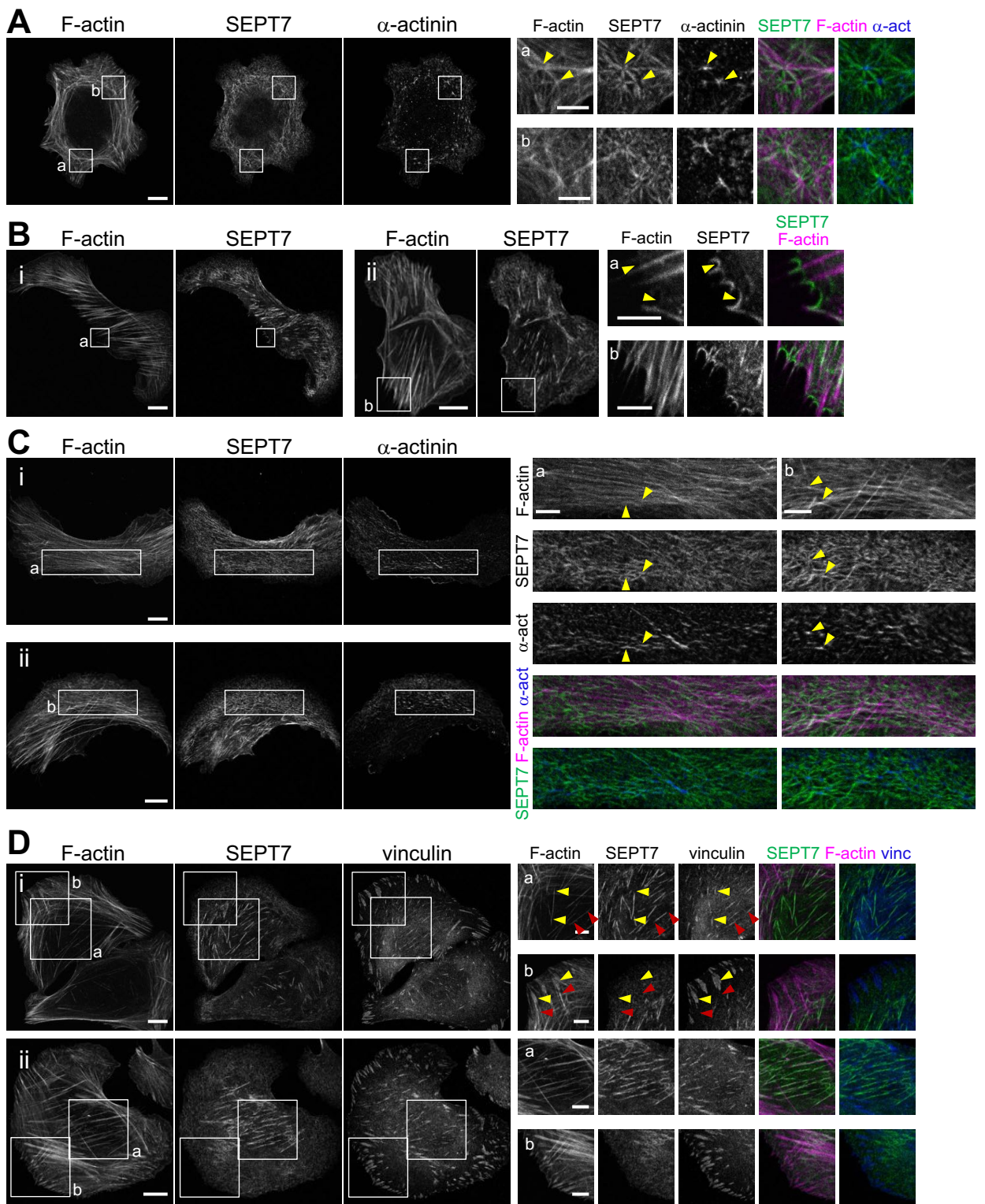
2277 D values inferior to $0.011 \mu\text{m}^2.\text{s}^{-1}$ corresponds to immobilized proteins. Values represent
2278 the average of the distributions obtained from different cells. (K) Fraction of mEos2-actin
2279 (blue), SEPT9_i3-mEos3.2 (magenta) and SEPT9_i3-mEos3.2-CAAX (light magenta)
2280 undergoing free diffusion, confined diffusion or immobilization outside FAs. Values
2281 represent the average of the fractions obtained from different cells (error bars: SEM).
2282 Results for SEPT9_i3-mEos3.2 (14 cells) correspond to pooled data from two
2283 independent experiments with n, the number of trajectories analyzed: SEPT9_i3-
2284 mEos3.2 $n_{\text{SEPT9_i3}} = 72,720$. Results for mEos2-actin (9 cells) and SEPT9_i3-mEos3.2-
2285 CAAX (5 cells) correspond each to data from one experiment with n, the number of
2286 trajectories analyzed: mEos2-actin $n_{\text{actin}} = 34,715$; SEPT9_i3-mEos3.2-CAAX $n_{\text{SEPT9_i3-}}$
2287 $n_{\text{CAAX}} = 37,339$. Statistical significance in (K) was obtained using two-tailed, non-parametric
2288 Mann–Whitney rank sum test. The different conditions were compared to the SEPT9_i3-
2289 mEos3.2 condition. The resulting P values are indicated as follows: *** $P < 0.001$; ****
2290 $P < 0.0001$. (L) Working model supported by the results of this study. Septins in cells
2291 organize as paired, octamer-based filaments mediating actin-membrane anchoring.



2292 **Figure 8. Septins on microtubules organize as octamer-based filaments. (A)**
2293 Representative confocal micrographs of SEPT7 (i) SEPT9 (ii) immunostained cells co-
2294 stained for microtubules (α -tubulin). Examples show septins localizing to microtubules
2295 close to the microtubule organizing center. **(B)** Representative confocal micrographs of
2296 SEPT9_i1-SEPT9_i1 reconstituted GFP (rGFP) distribution in fixed cells (i, ii) co-stained
2297 for microtubules (α -tubulin). **(C)** Representative confocal micrographs of SEPT2-SEPT2
2298 rGFP distribution in live cells (i, ii) co-expressing mCherry-SEPT9_i1 (not shown) and
2299 labeled for microtubules (SiR-tubulin). **(D)** Representative confocal micrograph of a
2300 GFP1-9 cell co-expressing SEPT2NCmut- β 10 and - β 11, mCherry-SEPT9_i1 (not shown)
2301 and labeled for microtubules (SiR-tubulin), showing a diffuse cytosolic phenotype. **(E)**
2302 Representative confocal micrographs of SEPT7-SEPT7 rGFP distribution in live cells (i,
2303 ii) co-expressing mCherry-SEPT9_i1 (not shown) and labeled for microtubules (SiR-
2304 tubulin). **(F)** Representative example of a GFP1-9 cell co-expressing β 10- and β 11-
2305 SEPT7Gmut2 co-expressing mCherry-SEPT9_i1 (not shown) and labeled for
2306 microtubules (SiR-tubulin). (A-F) Scale bars in large fields of views, 10 μ m. Scale bars in
2307 insets, 2 μ m. **(G)** Representative SIM micrographs of cells (i-iv) expressing mCherry-
2308 SEPT9_i1 (not shown) co-stained for SEPT7 and α -tubulin. Scale bars in large fields of
2309 views, 10 μ m. Scale bars in insets, 2 μ m. **(H)** Box plots depict the distributions of
2310 measured widths, as the full width at half maximum (FWHM), of microtubules (MT) and
2311 MT-associated septins (SEPT7). The data points are plotted on top of the respective box
2312 plots; data points correspond to width measurements at multiple positions along MT and
2313 septin fibers and in multiple MT and septin fibers per cell in a total of 8 cells. On each box,
2314 the central mark indicates the median, and the bottom and top edges of the box indicate
2315 the 25th and 75th percentiles, respectively. The whiskers extend to the minimum and
2316 maximum values. The number of measurements is $n = 128$ and 112 for MTs and septins,
2317 respectively. The respective median values are 128 nm and 119 nm for MTs and septins,
2318 respectively.

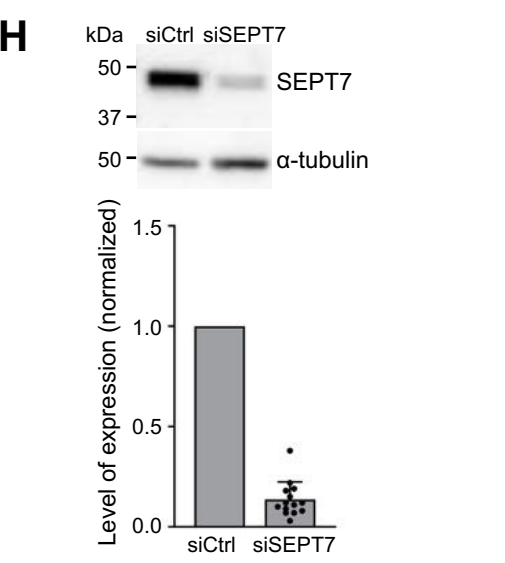
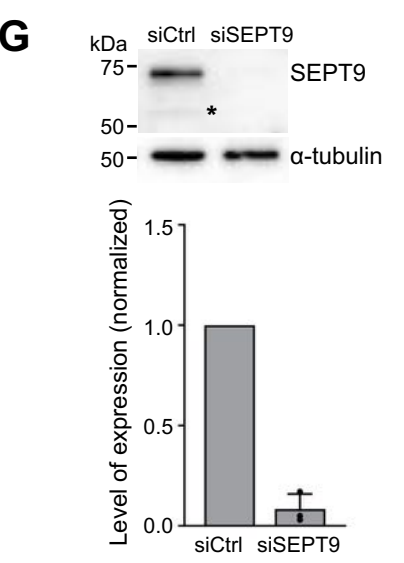
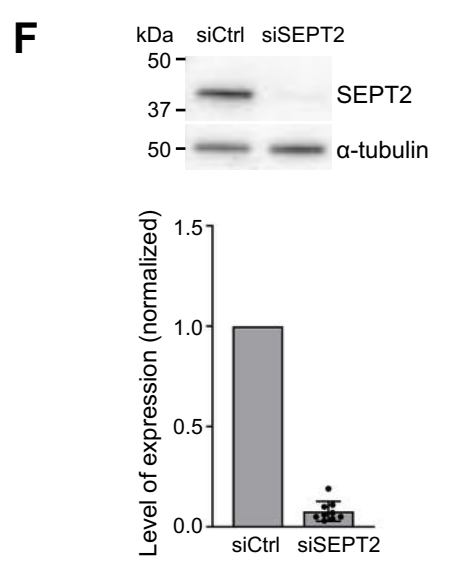
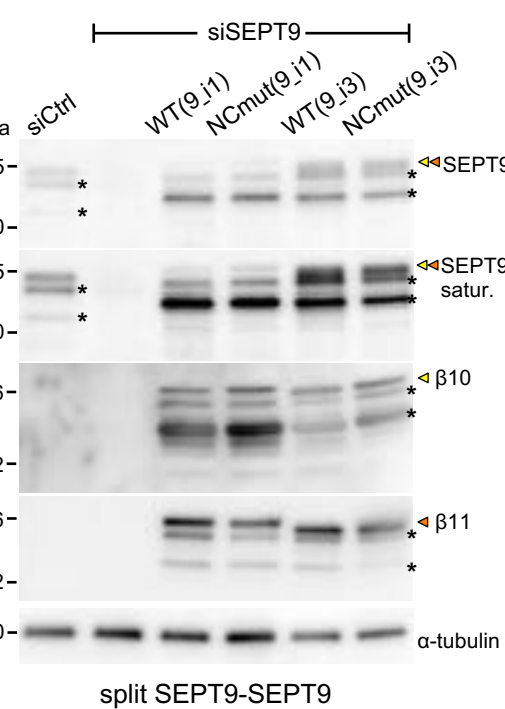
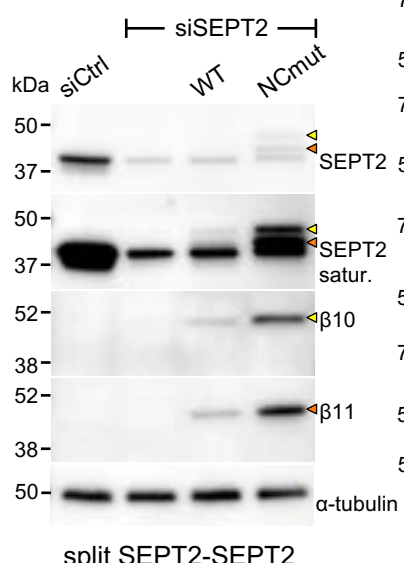
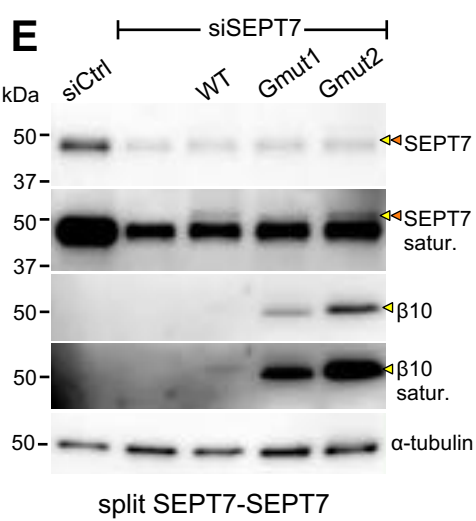
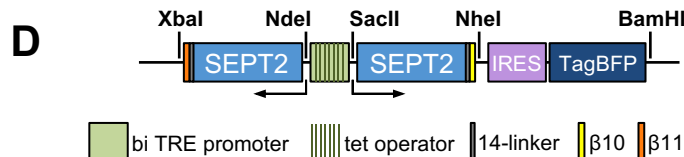
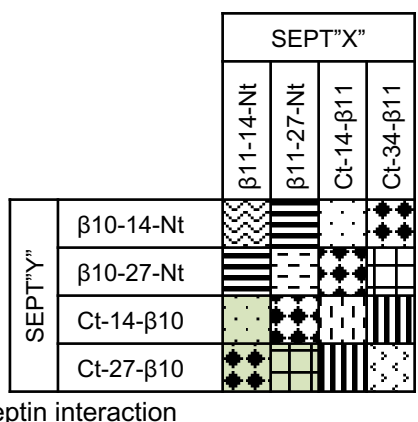
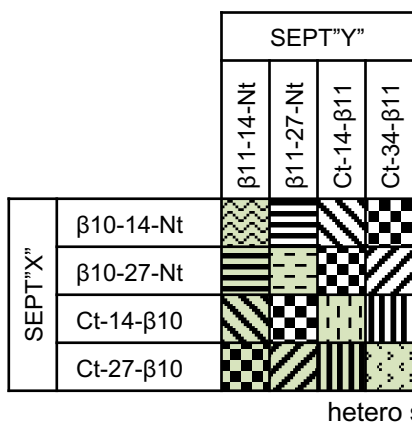
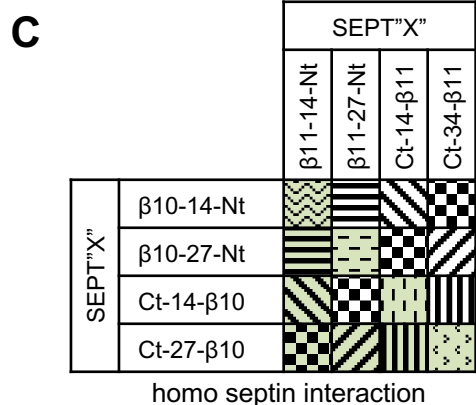
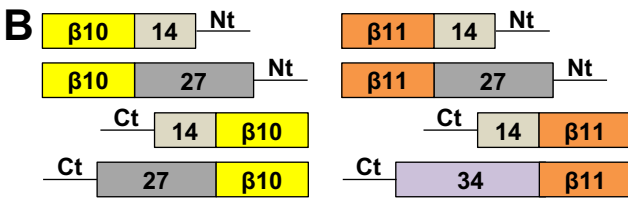


2319 **Figure S1. SEPT2 and SEPT9 distribution on different types of stress fibers in U2OS**
2320 **cells. (A)** Representative confocal micrographs of SEPT2 immunostained cells (i-iii) and
2321 cells expressing SEPT2-msfGFP (iv-vi). SEPT2 immunostained cells are co-stained for
2322 F-actin (phalloidin) and the FA protein paxillin. Examples show SEPT2 localizing (i) to
2323 peripheral (a) and ventral (b,c) SFs and excluded from focal adhesions (FA) (c), (ii) to
2324 peripheral (a) and perinuclear actin caps (b), (iii) to transverse arcs (b) and excluded from
2325 dorsal SFs (a,b), (iv) to ventral SFs (a,b), (v) to transverse arcs (a,b) and excluded from
2326 dorsal SFs (a), and (vi) showing a diffuse cytosolic phenotype. **(B)** Representative
2327 confocal micrographs of SEPT9 immunostained cells (i-iii) and cells expressing
2328 SEPT9_i3-msfGFP (iv-vi). SEPT9 immunostained cells are co-stained for F-actin
2329 (phalloidin) and the FA protein paxillin. Examples show SEPT9 localizing (i) to peripheral
2330 (a) and ventral (b,c) SFs and excluded from focal adhesions (FA) (c), (ii) to perinuclear
2331 actin caps (a,b), (iii) to transverse arcs (a) and ventral SFs (b), (iv) to transverse arcs (a)
2332 and excluded from dorsal SFs (a) and to ventral SFs (b), (v) to ventral SFs (a,b), and (vi)
2333 to peripheral (a) and perinuclear actin caps (b). Scale bars in large fields of views, 10 μm .
2334 Scale bars in insets, 2 μm . Related to Fig. 1B.



2335 **Figure S2. SEPT7 distribution on different types of stress fibers in U2OS cells. (A-**
2336 **D)** Representative confocal micrographs of SEPT7 immunostained cells co-stained for F-
2337 actin (phalloidin) (A-D), and additionally stained for α -actinin (A,C) or the FA protein
2338 vinculin (D). Examples show SEPT7 localization to ventral actin nodes (A), to arcs and
2339 arc nodes (C), and to ventral SFs, excluded from FAs (D). Yellow arrowheads in (A) point
2340 to two α -actinin-enriched actin nodes. Yellow arrowheads in (B) point to curved segments
2341 of the membrane that appear devoid of phalloidin staining, but positive for SEPT7. Yellow
2342 arrowheads in (C) point to α -actinin-enriched arc nodes. Yellow and red arrowheads in
2343 (Di, box a) point to the septin-decorated regions of two ventral SFs, with SEPT7 excluded
2344 from the respective FAs. Yellow and red arrowheads in (Di, box b) point to two dorsal
2345 SFs, with SEPT7 excluded from them and from the respective FAs. Scale bars in large
2346 fields of views, 10 μ m. Scale bars in insets, 5 μ m. Related to Fig. 1B.

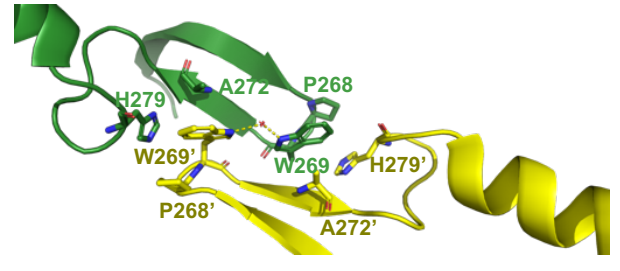
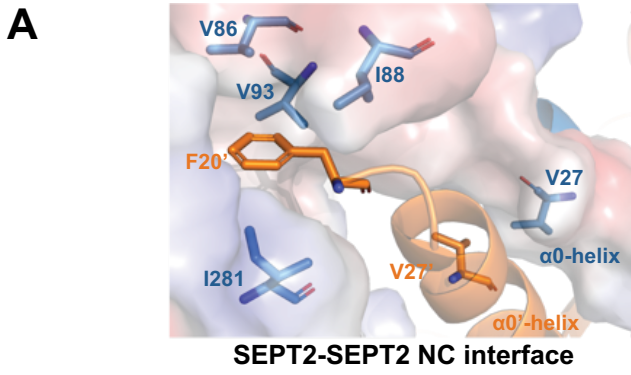
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 27-linker IDGAGGSPGGGSGGSGSGGGGSGGGGS
 14-linker IDGGGGSGGGGSSG
 β10 MGDLPDDHYLSTQTILSKDLN
 β11 EKRDHMLVLEYVTAAGITDAS



Martins et al Fig S3

2347 **Figure S3. Design of the tripartite split-GFP complementation assay for probing**
2348 **septin organization. (A)** Sequences of the β -10 and β 11-tags used for all split assays
2349 and of the linker sequences tested in screening experiments (B,C); 14-residue linkers
2350 were used throughout this study. **(B)** Schematic of N- and C-terminal β -10 and β 11-tag
2351 septin fusions tested in screening experiments (C) using short or long linkers (A). **(C)**
2352 Schematic of β -10 and β 11-septin fusion combinations for screening tripartite split GFP
2353 complementation. Combinations with the same pattern were considered to be equivalent
2354 (for example, SEPT2-14- β 10 / β 11-14-SEPT2 and SEPT2-14- β 11 / β 10-14-SEPT2). The
2355 combinations in green are the ones tested experimentally. **(D)** Schematic of the pTRIP
2356 TRE Bi vector bearing a bidirectional tetracycline response element (TRE) promoter for
2357 the doxycycline-inducible co-expression of β 10- and β 11-tagged septins. An IRES-
2358 TagBFP cassette was used for monitoring septin expression. Restriction sites used for
2359 subcloning are indicated (see methods for details). **(E)** Left, Western blots of U2OS-Tet-
2360 On-GFP1-9 cell line lysates probed with anti-SEPT7, anti- β 10 and anti- α -tubulin
2361 antibodies upon treatment with siRNAs targeting LacZ (siCtrl), SEPT7 (siSEPT7), and
2362 targeting SEPT7 while co-expressing wild-type β 10- and β 11-SEPT7 (WT), β 10- and β 11-
2363 SEPT7Gmut1 (Gmut1), and β 10- and β 11-SEPT7Gmut2 (Gmut2). Yellow and orange
2364 arrowheads point to bands correspond to β 10- and β 11-fusions. The SEPT7 and β 10 blots
2365 are also shown saturated in purpose for displaying weaker bands. Molecular weight
2366 markers are shown on the left. Middle, Western blots of U2OS-Tet-On-GFP1-9 cell line
2367 lysates probed with anti-SEPT2, anti- β 10, anti- β 11 and anti- α -tubulin antibodies upon
2368 treatment with siRNAs targeting LacZ (siCtrl), SEPT2 (siSEPT2), and targeting SEPT2
2369 while co-expressing wild-type SEPT2- β 10 and - β 11 (WT) or SEPT2NCmut- β 10 and - β 11
2370 (NCmut). Yellow and orange arrowheads point to bands correspond to β 10- and β 11-
2371 fusions. The SEPT2 blot is also shown saturated in purpose for displaying weaker bands.
2372 Right, Western blots of U2OS-Tet-On-GFP1-9 cell line lysates probed with anti-SEPT9,
2373 anti- β 10, anti- β 11 and anti- α -tubulin antibodies upon treatment with siRNAs targeting
2374 LacZ (siCtrl), SEPT9 (siSEPT9), and targeting SEPT9 while co-expressing wild-type
2375 SEPT9- β 10 and - β 11 (WT) or SEPT9NCmut- β 10 and - β 11 (NCmut) for both SEPT9_i1
2376 and SEPT9_i3. Yellow and orange arrowheads point to bands correspond to β 10- and
2377 β 11-fusions. The SEPT9 blot is also shown saturated in purpose for displaying weaker
2378 bands. Asterisks point to SEPT9 degradation products. **(F)** Western blot of U2OS cell
2379 lysates probed with anti-SEPT2 and anti- α -tubulin antibodies upon treatment with siRNAs
2380 targeting LacZ (siCtrl) or SEPT2 (siSEPT2). Molecular weight markers are shown on the
2381 left. Bottom, respective quantification of SEPT2 protein levels (mean+SD). Mean values
2382 (normalized to 1 for siCtrl) are from 3 independent siCtrl and 9 independent siSEPT2
2383 treatments. SEPT2 was knocked down on average by 92%. **(G)** Same as (F) for SEPT9.
2384 The asterisk points to a SEPT9 degradation product. Mean values (normalized to 1 for
2385 siCtrl) are from 3 independent siCtrl and 3 independent siSEPT9 treatments. SEPT9 was
2386 knocked down on average by 92%. **(H)** Same as (F) for SEPT7. Mean values (normalized

2387 to 1 for siCtrl) are from 3 independent siCtrl and 12 independent siSEPT7 treatments.
2388 SEPT7 was knocked down on average by 86%.



	$\alpha 0$ -helix			$\beta 2$		$\alpha 5$	
SEPT2	20	27		86 88	93		281
	G <u>F</u> ANLPNO <u>V</u> HRKSVKKG... <u>E</u> AST <u>V</u> E <u>I</u> EERG <u>V</u> KLR... <u>L</u> RT <u>M</u> L <u>I</u>						
SEPT9 _{j3}	263	270		329331	336		523
	G <u>I</u> DSILEQ <u>M</u> RRKAMKQG... <u>K</u> SIT <u>H</u> D <u>I</u> E <u>E</u> K <u>G</u> V <u>R</u> MK... <u>L</u> R <u>D</u> LL <u>I</u>						
	*:	:	:	:	:	:	:
	:	:	:	:	:	:	:
	:	:	:	:	:	:	:

	$\beta 7$	$\beta 8$	
SEPT7	269	272	279
	Q <u>Y</u> P <u>W</u> G <u>V</u> A <u>E</u> V <u>E</u> N <u>G</u> E <u>H</u>		
SEPT9 _{j3}	502	505	512
	K <u>T</u> K <u>W</u> G <u>T</u> I <u>E</u> V <u>E</u> N <u>T</u> T <u>H</u>		
	**	*	*

NC interface mutants

SEPT2NCmut F20D, V27D

SEPT9_{j1}NCmut I281D, M288D

SEPT9_{j3}NCmut I263D, M270D

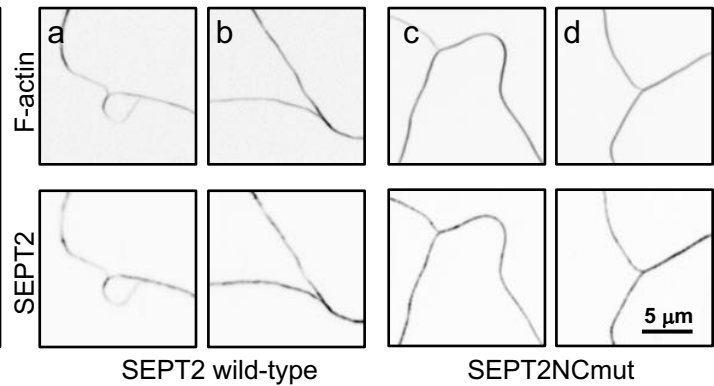
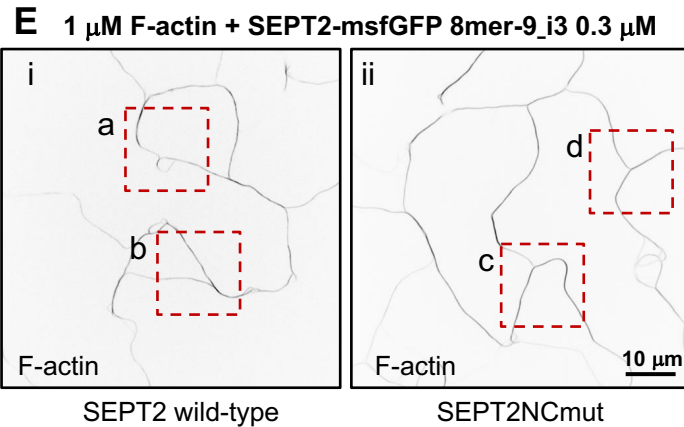
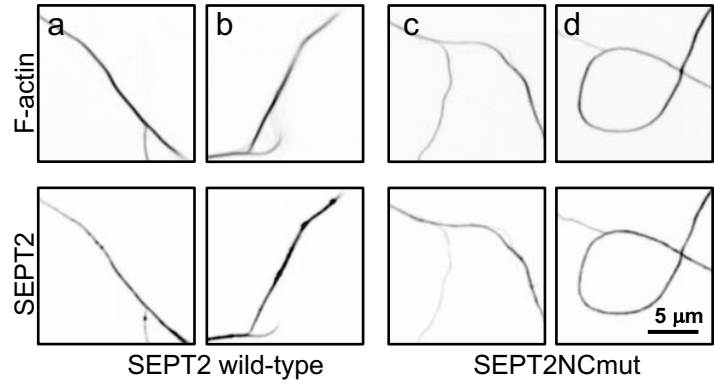
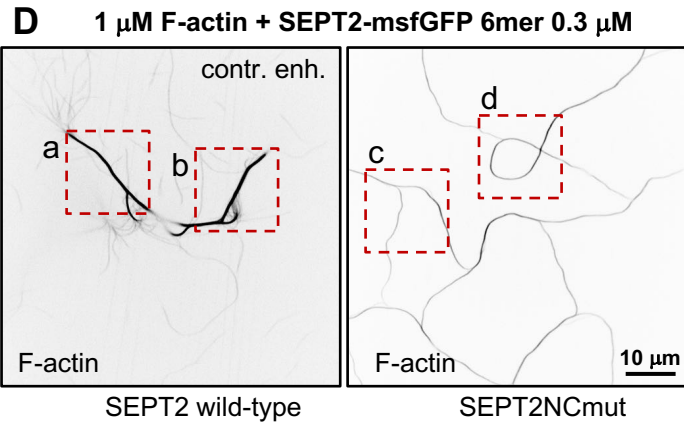
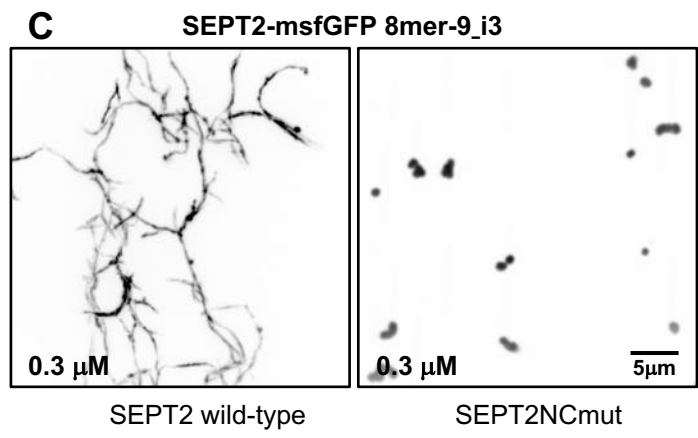
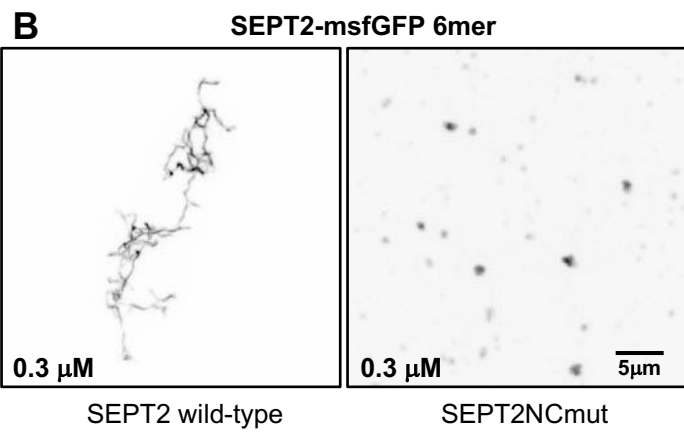
G interface mutants

SEPT7Gmut1 W269A, H279D

SEPT7Gmut2 H279D

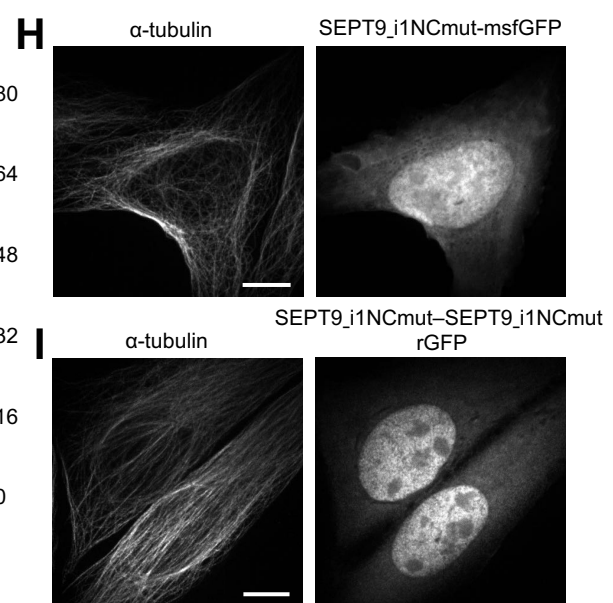
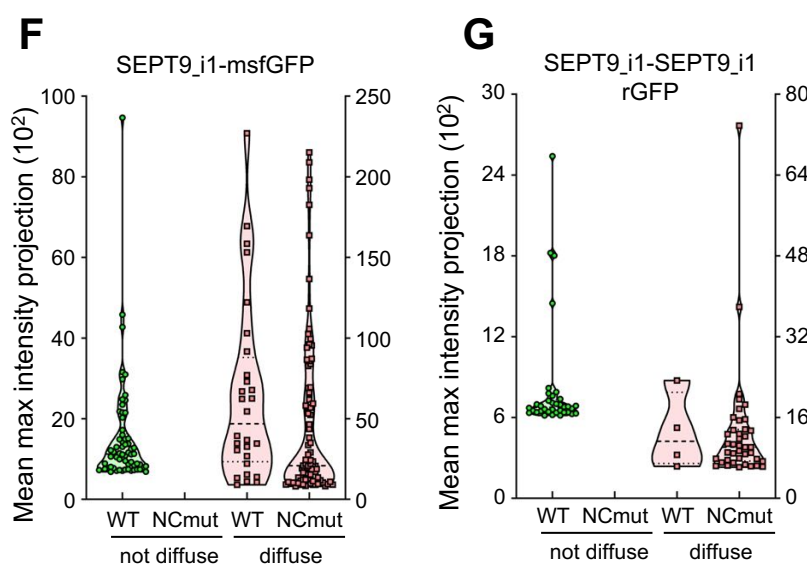
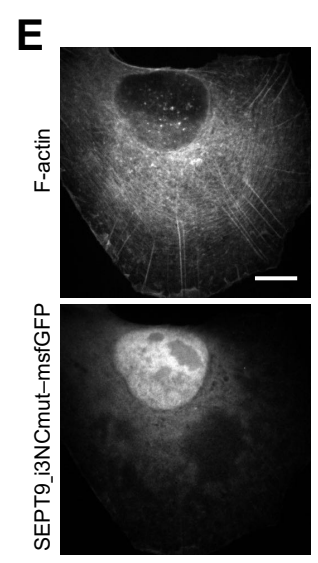
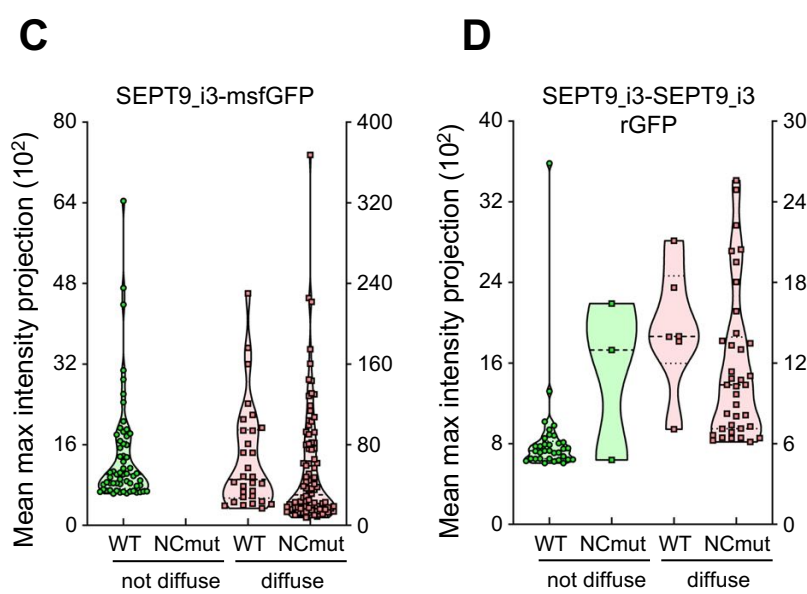
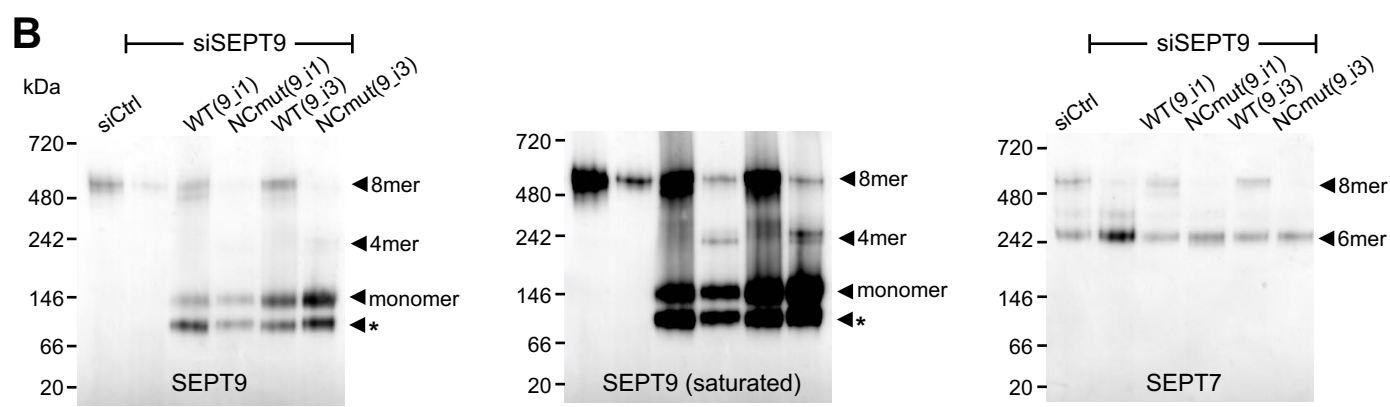
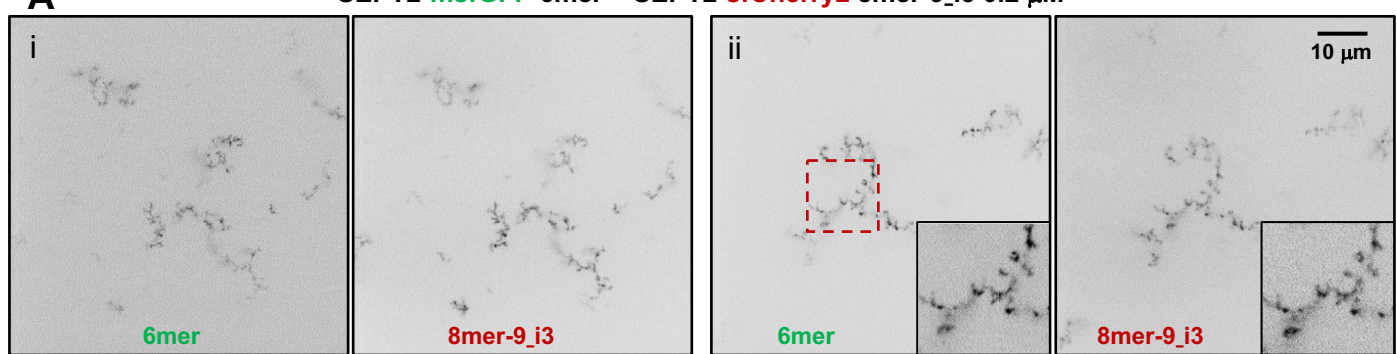
SEPT9_{i1}Gmut W520A, H530D

SEPT9_{i3}Gmut W502A, H512D

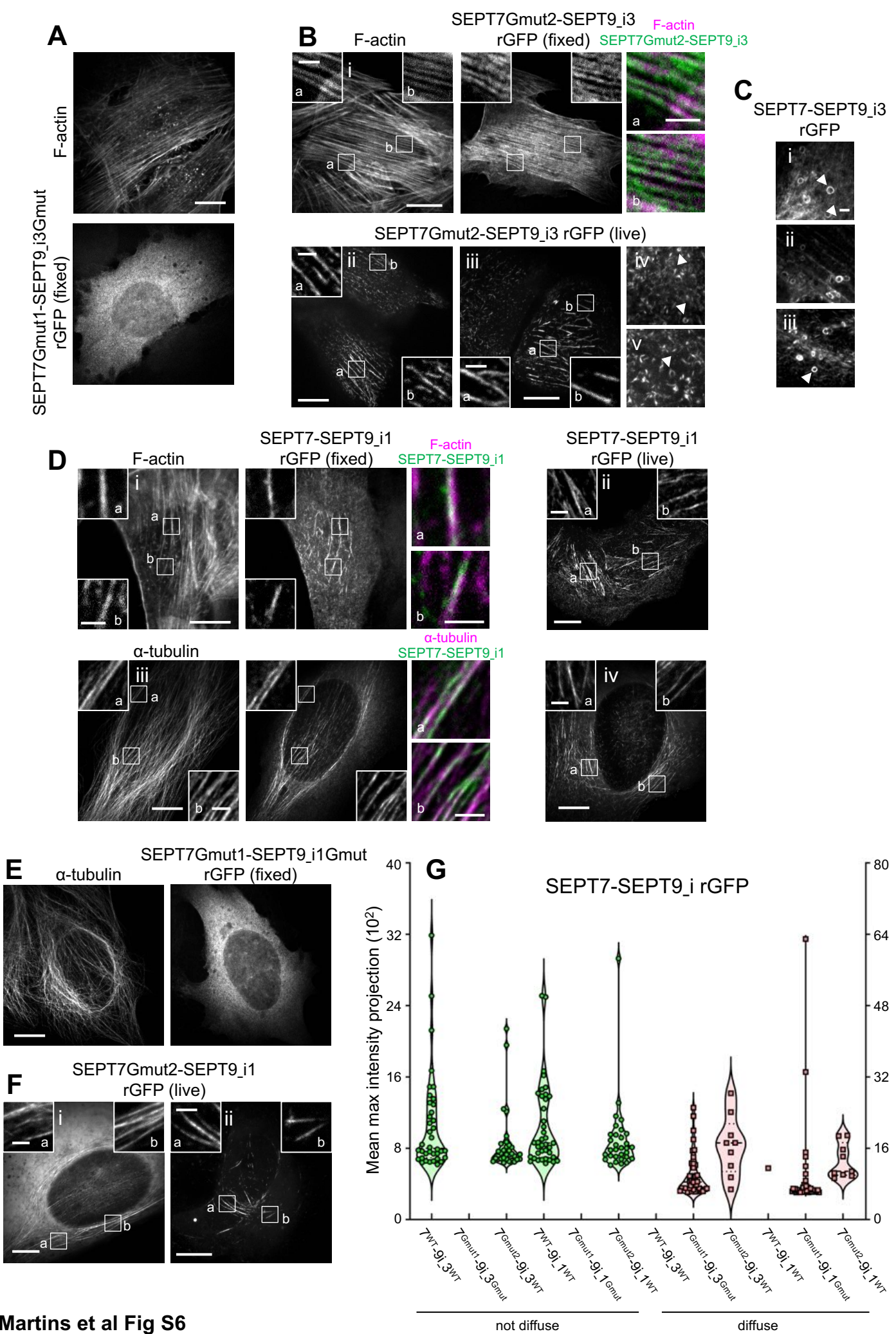


2389 **Figure S4. Septin interface mutants used in this study and cell-free reconstitution**
2390 **of septin assembly. (A)** Left, Top, conserved residues in the SEPT2-SEPT2 NC
2391 interface are shown in the crystal structure of human SEPT2 homodimers (PDB 2QA5)
2392 (Sirajuddin et al., 2007). The backbone structure is displayed as a cartoon representation
2393 in PyMOL, with critical residues represented as sticks (deep blue and red for nitrogen and
2394 oxygen atoms, respectively). Residues F20 from the hook-loop of one SEPT2 subunit
2395 (orange) interact with the hydrophobic cleft formed by V86, I88, V93, and I281 of the
2396 adjacent SEPT2 subunit (blue). The importance of this phenylalanine in anchoring the $\alpha 0$
2397 helix at the NC interface was emphasized only recently (Cavini et al., 2021). The blue
2398 subunit's surface representation highlights the complementary of shape between the two
2399 SEPT2 in this interface. The interaction between the $\alpha 0$ helices of each subunit is also
2400 stabilized via a hydrophobic interaction between their respective V27. Left, Middle,
2401 sequence alignment of the regions including the residues shown in the NC interface
2402 structure for SEPT2 and SEPT9_i3. The structural elements ($\alpha 0$, $\beta 2$, $\alpha 5$) related to these
2403 residues are underlined and shown above the sequences. The consensus symbols are
2404 from ClustalW alignments of all human septins (*, fully conserved residue; colon,
2405 conservation between residues with strongly similar physicochemical properties; period,
2406 conservation between residues with weakly similar physicochemical properties). We note
2407 that the residues described above are strictly or physicochemically conserved (except for
2408 V86), highlighting their importance in stabilizing the SEPT2-SEPT2 NC interface. Left,
2409 Bottom, NC interface mutants used in this study. A mutation of F20D/I263D is expected
2410 to destabilize the hydrophobic pocket depicted above, whereas a V27D/M270D is
2411 expected to destabilize the $\alpha 0$ helices interface. Importantly, a strictly conserved
2412 aspartate (SEPT2 E90, corresponding to SEPT6 E90 and SEPT7 E102 which are well
2413 defined in the cryo-EM structure of the SEPT6-SEPT7 NC interface (Mendonca et al.,
2414 2021)) in the loop connecting $\beta 2$ and $\beta 3$ is pointing to the hydrophobic cleft where the
2415 phenylalanine resides. The F20D mutation is thus expected to result in a repulsion
2416 between the aspartate and glutamate and contribute further to the destabilization of the
2417 NC interface. Right, Top, conserved residues in the SEPT7-SEPT7 G interface are shown
2418 in the crystal structure of human SEPT7 homodimers (PDB 6N0B) (Brognara et al., 2019).
2419 The backbone structure is displayed as a cartoon representation in PyMOL, with critical
2420 residues represented as sticks (deep blue and red for nitrogen and oxygen atoms,
2421 respectively). Residues W269 of one SEPT7 subunit (yellow) interact with residues
2422 W269, A272 and H279 in the adjacent SEPT7 subunit (green) (Sirajuddin et al., 2007;
2423 Zent et al., 2011). W269 from adjacent subunits interact via a water molecule bridge
2424 through hydrogen bonds. In addition, each W269 is engaged in π - π interactions with H279
2425 and CH- π interactions with A272 of the opposite subunit. Right, Middle, sequence
2426 alignment of the regions including the residues shown in the G interface structure for
2427 SEPT7 and SEPT9_i3. The structural elements ($\beta 7$, $\beta 8$) related to these residues are
2428 underlined and shown above the sequences. The consensus symbols are from ClustalW
2429 alignments of all human septins (*, fully conserved residue; colon, conservation between

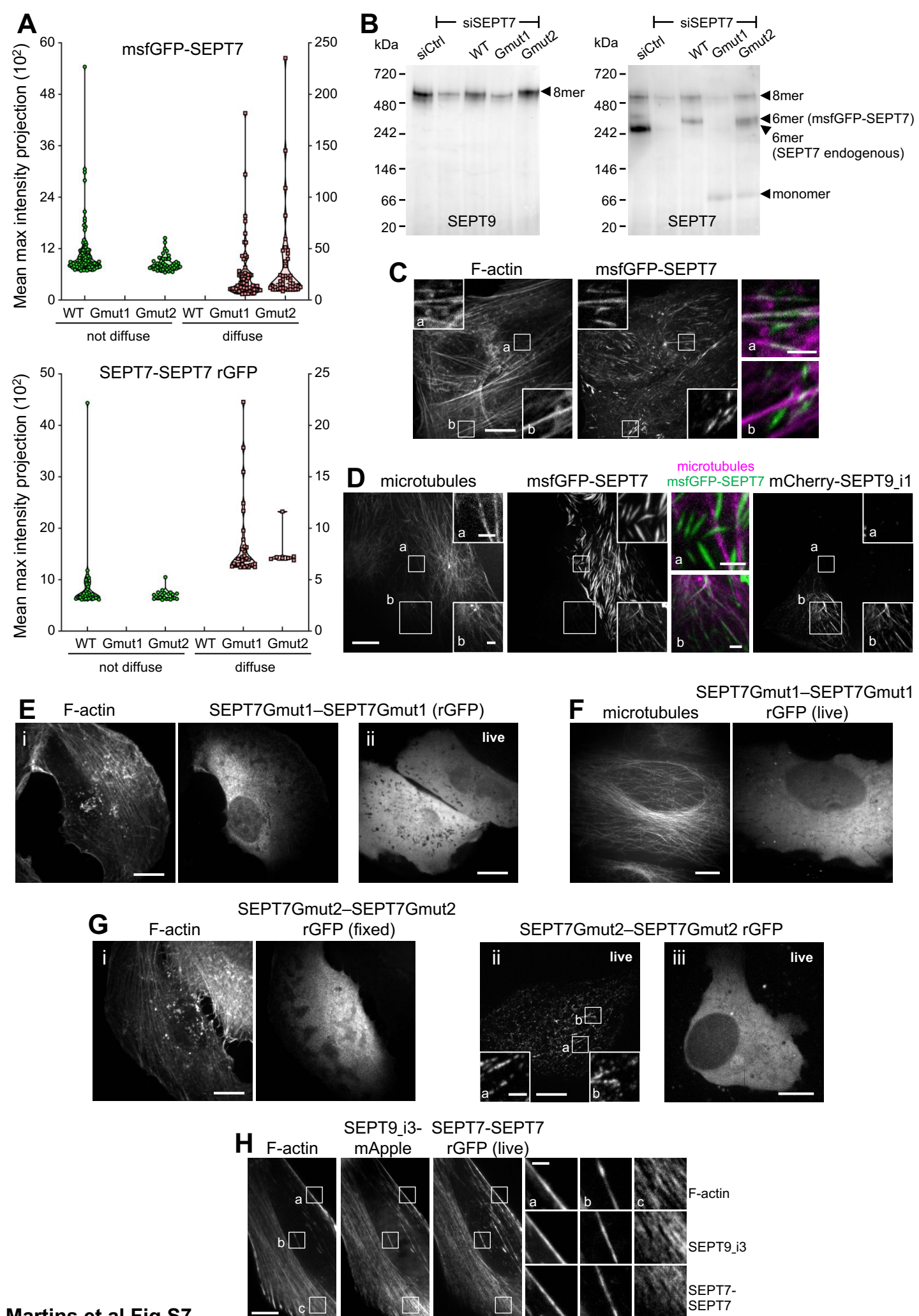
2430 residues with strongly similar physicochemical properties). Notice that W269 and H279
2431 are both strictly conserved, showing their importance in stabilizing this interface. Right,
2432 Bottom, G interface mutants were used in this study. The presence of both mutations
2433 W269A and H279D in SEPT7 and SEPT9 is expected to destabilize the SEPT7-SEPT7
2434 and SEPT7-SEPT9 G-interfaces. The loss of the aromatic cycle properties in the mutant
2435 W269A does not allow the abovementioned critical interactions mediated by the wild-type
2436 Trp. W269A is expected to destabilize H279 and potentially change its orientation. In
2437 addition, the much smaller size of the alanine will poorly mimic the hydrophobic interaction
2438 between W269 and H279, weakening the G-interface. Note that W269 is in the vicinity of
2439 Tyr267 of the same subunit. This tyrosine interacts with the nucleotide buried within the
2440 G-interface. Consequently, any mutations destabilizing W269 could dramatically
2441 destabilize the overall G-interface because of a domino effect. Similarly, H279D is
2442 expected to preclude hydrophobic interactions with W269 and thus destabilize the latter.
2443 The single mutation H279D in SEPT7 is expected to destabilize the SEPT7-SEPT7 G-
2444 interface when present in both SEPT7 subunits, but not the SEPT7-SEPT9 interface with
2445 wild-type SEPT9. **(B-C)** Representative spinning disk fluorescence images of septin
2446 filament assembly upon polymerization of hexamers (B) and octamers-9_i3 (C) at the
2447 indicated final protomer concentration. Protomers contained either wild-type SEPT2 (left
2448 panels) or SEPT2NCmut (right panels). Images use an inverted grayscale. Related to Fig.
2449 2F. **(D-E)** Representative spinning disk fluorescence images of reconstituted actin
2450 filaments, polymerizing in the presence of septin hexamers (D) and septin octamers (E).
2451 Protomers contained either wild-type SEPT2 or SEPT2NCmut. Actin filaments are
2452 visualized with AlexaFluor568-conjugated phalloidin, and septins with SEPT2-msfGFP.
2453 One example of large fields of view are shown for each condition, depicting cross-linking
2454 of actin filaments; only actin labeling is shown. The image for actin in the presence of
2455 wild-type hexamers is contrast-enhanced in purpose in order to saturate the actin bundles
2456 so that weaker-intensity single actin filaments are also visible. Insets on the right side of
2457 each panel show higher magnifications of selected regions of interest on the left (dashed
2458 squares in red). Two regions of interest (a,b for wild-type SEPT2 and c,d for
2459 SEPT2NCmut) are shown in each case, depicting both the actin (top row) and septin
2460 (bottom row) signals. Scale bars in all large fields of views, 10 μm . Scale bars in all insets,
2461 5 μm .



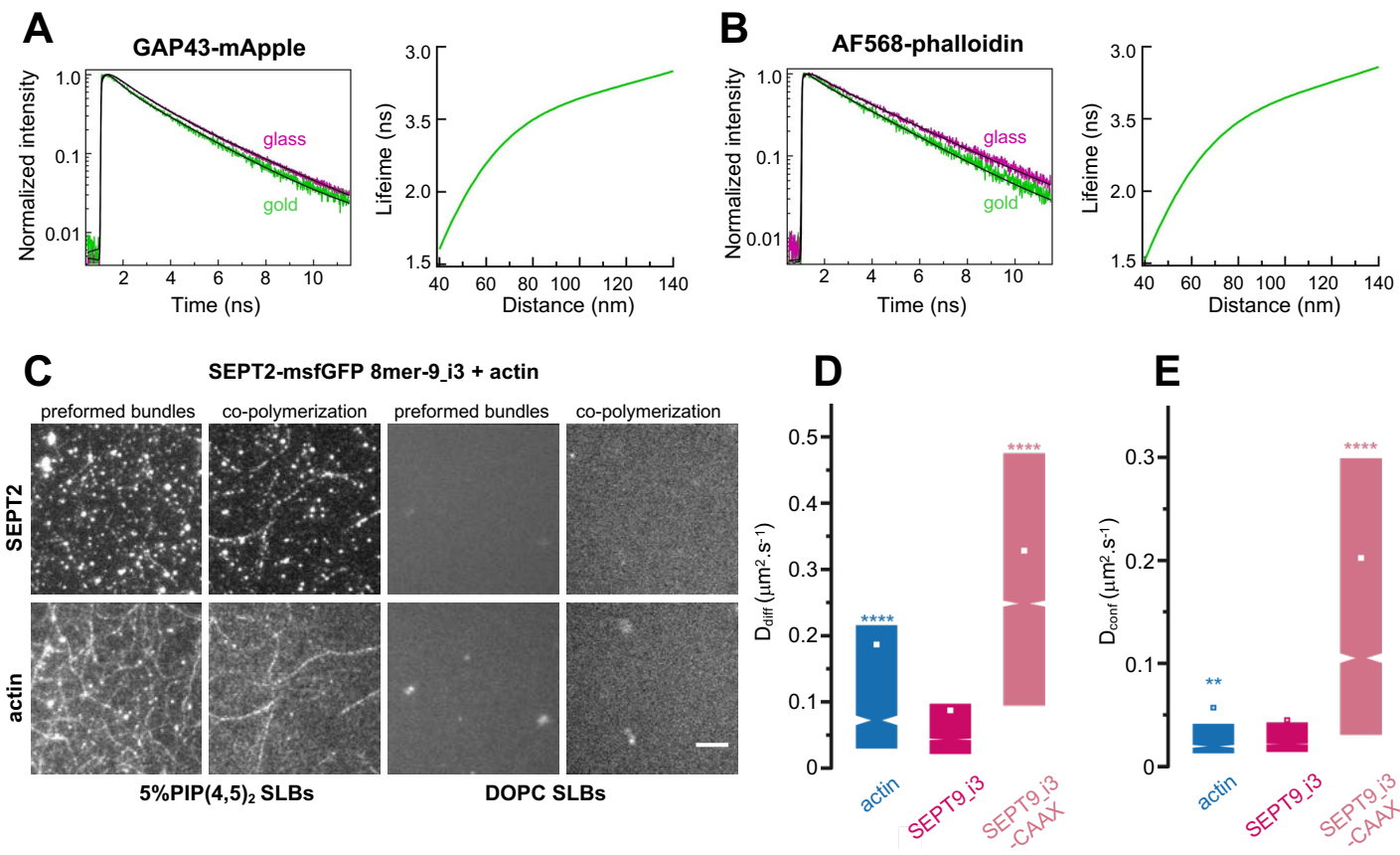
2462 **Figure S5. SEPT9 NC mutants used for split SEPT9-SEPT9 assays. (A)**
2463 Representative spinning disk fluorescence images of septin filament assembly upon co-
2464 polymerization of hexamers containing SEPT2-msfGFP and octamers-9_i3 containing
2465 SEPT2-sfCherry2 at the indicated final protomer concentration. Images use an inverted
2466 grayscale. Scale bars in all large fields of views, 10 μm . **(B)** Western blot following native
2467 PAGE of U2OS cell lysates probed with anti-SEPT9 (left and middle) and anti-SEPT7
2468 (right) antibodies upon treatment with siRNAs targeting LacZ (siCtrl), SEPT9 (siSEPT9),
2469 and targeting SEPT9 while expressing wild-type SEPT9-msfGFP (WT) or SEPT9NCmut-
2470 msfGFP (NCmut) for both SEPT9_i1 and SEPT9_i3. The SEPT9 blot is also shown
2471 saturated in purpose for displaying weaker bands. Arrowheads point to the sizes of the
2472 indicated complexes. The asterisks point to SEPT9 degradation. Molecular weight
2473 markers are shown on the left. **(C)** Violin plots depicting the distribution of diffuse cytosolic
2474 (red datapoints) vs. non-diffuse (green datapoints) phenotypes in cells expressing wild-
2475 type SEPT9_i3-msfGFP or SEPT9_i3NCmut-msfGFP. Data points are from a total of 90
2476 cells each for wild-type and mutant SEPT9 distributed among the two phenotypes. **(D)**
2477 Violin plots depicting the distribution of diffuse cytosolic (red datapoints) vs. non-diffuse
2478 (green datapoints) phenotypes from reconstituted GFP (rGFP) in GFP1-9 cells co-
2479 expressing wild-type SEPT9_i3- β 10 and - β 11 or SEPT9_i3NCmut- β 10 and - β 11. Data
2480 points are from a total of 40 cells each for wild-type and mutant SEPT9 distributed among
2481 the two phenotypes. **(E)** Representative example of a cell expressing SEPT9_i3NCmut-
2482 msfGFP showing a diffuse cytosolic phenotype. Scale bar, 10 μm . **(F-H)** Same as (C-E)
2483 for SEPT9_i1. **(I)** Representative example of a GFP1-9 cell co-expressing
2484 SEPT9_i1NCmut- β 10 and - β 11 showing a diffuse cytosolic phenotype. Scale bar, 10 μm .
2485 Related to Fig. 3C,E.



2486 **Figure S6. SEPT7 and SEPT9 G mutants used for split SEPT7-SEPT9 assays. (A)**
2487 Representative example of a GFP1-9 cell co-expressing β 11-SEPT7Gmut1 and
2488 SEPT9_i3Gmut- β 10 showing a diffuse cytosolic phenotype. The cell is co-stained for F-
2489 actin (phalloidin). Scale bar, 10 μ m. **(B)** Representative examples of GFP1-9 cells (i-v)
2490 co-expressing β 11-SEPT7Gmut2 and SEPT9_i3- β 10. The fixed cell is co-stained for F-
2491 actin (phalloidin). Examples show reconstituted GFP (rGFP) localizing (i) to ventral SFs
2492 (a,b), (ii) to perinuclear actin caps (a,b), (iii) to ventral SFs (a,b), and (iv-v) rings
2493 (arrowheads). Scale bars in large fields of views, 10 μ m. Scale bars in insets, 2 μ m. **(C)**
2494 Examples of rings (arrowheads) in GFP1-9 cells co-expressing β 11-SEPT7 and
2495 SEPT9_i3- β 10. Scale bar, 2 μ m. **(D)** Representative examples of GFP1-9 cells co-
2496 expressing β 11-SEPT7 and SEPT9_i1- β 10 (i-iv). The fixed cells are co-stained for F-actin
2497 (phalloidin) (i) or for α -tubulin (iii). Examples show rGFP localizing to ventral SFs (i,ii) or
2498 to microtubules (iii,iv). Scale bars in large fields of views, 10 μ m. Scale bars in insets, 2
2499 μ m. **(E)** Representative example of GFP1-9 cell co-expressing β 11-SEPT7Gmut1 and
2500 SEPT9_i1Gmut- β 10 co-stained for α -tubulin showing a diffuse cytosolic phenotype. Scale
2501 bar, 10 μ m. **(F)** Representative examples of GFP1-9 cells co-expressing β 11-
2502 SEPT7Gmut2 and SEPT9_i1- β 10 with localizing to microtubules. Scale bars in large
2503 fields of views, 10 μ m. Scale bars in insets, 2 μ m. **(G)** Violin plots depicting the distribution
2504 of diffuse cytosolic (red datapoints) vs. non-diffuse (green datapoints) phenotypes in
2505 GFP1-9 cells co-expressing the indicated combinations of β 11-SEPT7 and SEPT9- β 10
2506 fusions. Data points are from a total of 40 cells each for each combination, distributed
2507 among the two phenotypes. Related to Fig. 3D.



2508 Figure S7. **SEPT7 G mutants used for split SEPT7-SEPT7 assays.** (A) Top, Violin plots
2509 depicting the distribution of diffuse cytosolic (red datapoints) vs. non-diffuse (green
2510 datapoints) phenotypes in cells expressing wild-type msfGFP-SEPT7 or msfGFP-
2511 SEPT7NCmut. Data points are from a total of 71 cells for wild-type, 68 cells for
2512 SEPT7Gmut1 and 90 cells for SEPT7Gmut2 distributed among the two phenotypes.
2513 Bottom, Violin plots depicting the distribution of diffuse cytosolic (red datapoints) vs. non-
2514 diffuse (green datapoints) phenotypes from reconstituted GFP (rGFP) in GFP1-9 cells co-
2515 expressing wild-type β 10- and β 11-SEPT7, β 10- and β 11-SEPT7Gmut1, or β 10- and β 11-
2516 SEPT7Gmut2. Data points are from a total of 40 cells for wild-type, 33 cells for β 10- and
2517 β 11-SEPT7Gmut1 and 29 cells for β 10- and β 11-SEPT7Gmut2 distributed among the two
2518 phenotypes. (B) Western blot following native PAGE of U2OS cell lysates probed with
2519 anti-SEPT9 (left) and anti-SEPT7 (right) antibodies upon treatment with siRNAs targeting
2520 LacZ (siCtrl), SEPT7 (siSEPT7), and targeting SEPT7 while expressing wild-type
2521 msfGFP-SEPT7 (WT), msfGFP-SEPT7Gmut1 (Gmut1), or msfGFP-SEPT7Gmut2
2522 (Gmut2). Arrowheads point to the sizes of the indicated complexes. Molecular weight
2523 markers are shown on the left. (C) Representative confocal micrograph of a cell
2524 expressing msfGFP-SEPT7 co-stained for F-actin (phalloidin) localizing to ventral SFs (a)
2525 and to ectopic bundles devoid of phalloidin staining (b). Scale bars in large fields of views,
2526 10 μ m. Scale bars in insets, 2 μ m. Related to Fig. 4A. (D) Representative example of a
2527 cell (bottom left) co-expressing msfGFP-SEPT7 and mCherry1-SEPT9_i1 and labeled for
2528 microtubules (SiR-tubulin) showing msfGFP-SEPT7 localizing to microtubules (b). A cell
2529 expressing only msfGFP-SEPT7 (top right) shows msfGFP-SEPT7 localizing to ectopic
2530 bundles not co-localizing with microtubules (a). Scale bars in large fields of views, 10 μ m.
2531 Scale bars in insets, 2 μ m. Related to Fig. 4G. (E) Representative examples of GFP1-9
2532 cells (i,ii) co-expressing β 10- and β 11-SEPT7Gmut1 showing a diffuse cytosolic
2533 phenotype. The fixed cell is co-stained for F-actin (phalloidin). Scale bar, 10 μ m. (F)
2534 Representative example of GFP1-9 cells co-expressing β 10- and β 11-SEPT7Gmut1,
2535 mCherry-SEPT9_i1 (not shown) and labeled for microtubules (SiR-tubulin) showing a
2536 diffuse cytosolic phenotype. Scale bar, 10 μ m. (G) Representative examples of GFP1-9
2537 cells (i-iii) co-expressing β 10- and β 11-SEPT7Gmut2. The fixed cell is co-stained for F-
2538 actin (phalloidin). Examples show diffuse cytosolic phenotypes (i,iii) of the rGFP and rGFP
2539 localizing to SFs (ii). Scale bars in large fields of views, 10 μ m. Scale bars in insets, 2 μ m.
2540 (H) Representative example of a GFP1-9 cell co-expressing β 10- and β 11-SEPT7,
2541 SEPT9_i3-mApple and labeled for F-actin (SiR-actin). Example shows rGFP localization
2542 to peripheral (a), ventral SFs (b) and transverse arcs (c). Scale bars in large fields of
2543 views, 10 μ m. Scale bars in insets, 2 μ m. Related to Fig. 4F.



2544 **Figure S8. Septin filaments are closely apposed to the plasma membrane, are**
2545 **immobilized on actin stress fibers, and can mediate actin-membrane anchoring. (A-**
2546 **B)** depict representative examples of lifetime decay traces for GAP43-mApple (A, left)
2547 and AF568-phalloidin (F-actin) (B, left) on glass and in the presence of gold. The solid
2548 lines represent the numerical fits, showing the lifetime reduction due to the MIET process.
2549 The respective calculated lifetime-distance dependences, used to calculate the distances
2550 of the fluorophores from the coverslip (Fig. 7E) are shown in the respective right panels.
2551 Related to Fig. 7B-E. **(C)** TIRF images of SEPT2-msfGFP 8mer-9_i3 and F-actin, either
2552 co-polymerized on top of a supported lipid bilayer (SLB), or co-polymerized in solution to
2553 form preformed bundles that were then flushed onto the supported lipid bilayer. The
2554 supported lipid bilayer was composed either of 5% of PI(4,5)P₂, a septin-interacting lipid,
2555 and 95% DOPC (top panels), or 100% DOPC (bottom panels). Scale bar, 5µm. Related
2556 to Fig. 7F. **(D-E)** Box plots displaying the median (notch) and mean (square) ± percentile
2557 (25–75%) of diffusion coefficients corresponding to the free diffusion (D_{diff}) (D) and
2558 confined diffusion (D_{conf}) (E) trajectories outside FAs from sptPALM. Related to Fig. 7G-
2559 K. Statistical significance was obtained using two-tailed, non-parametric Mann–Whitney
2560 rank sum test. The different conditions were compared to the SEPT9_i3-mEos3.2
2561 condition. The resulting P values are indicated as follows: ** P<0.01; **** P < 0.0001.