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1 **Title**

2 **Human septins organize as octamer-based filaments and mediate**
3 **actin-membrane anchoring in cells**

4
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37
38 **Summary**
39 Martins et al. show that all septins associated with actin stress fibers organize as octamer-
40 based filaments that mediate actin-membrane anchoring. Depleting octamers or
41 preventing septins from polymerizing leads to a partial loss of stress fibers and
42 compromised cell mechanics.

43
44 **Abstract**

45 Septins are cytoskeletal proteins conserved from algae and protists to mammals. A
46 unique feature of septins is their presence as heteromeric complexes that polymerize into
47 filaments in solution and on lipid membranes. Although animal septins associate
48 extensively with actin-based structures in cells, whether septins organize as filaments in
49 cells and if septin organization impacts septin function is not known. Customizing a
50 tripartite split-GFP complementation assay, we show that all septins decorating actin
51 stress fibers are octamer-containing filaments. Depleting octamers or preventing septins
52 from polymerizing leads to a loss of stress fibers and reduced cell stiffness. Super-
53 resolution microscopy revealed septin fibers with widths compatible with their
54 organization as paired septin filaments. Nanometer-resolved distance measurements and
55 single-protein tracking further showed that septin filaments are membrane-bound and
56 largely immobilized. Finally, reconstitution assays showed that septin filaments mediate
57 actin-membrane anchoring. We propose that septin organization as octamer-based
58 filaments is essential for septin function in anchoring and stabilizing actin filaments at the
59 plasma membrane.

60

61 **Introduction**

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

79 Biochemical isolation of native septins revealed that septins exist as stable
80 heteromeric complexes that can polymerize into filaments (Field et al., 1996; Frazier et
81 al., 1998; Hsu et al., 1998; Kim et al., 2011; Kinoshita et al., 2002; Sellin et al., 2011). The
82 isolation of recombinant septin complexes established that septin complexes are
83 palindromes, with each septin in two copies and in a specific position within the complex.
84 Each monomer interacts with its neighbors by alternating interfaces, named NC (from the
85 N- and C-terminal domains) and G (from the GTP-binding domain) (Bertin et al., 2008;

86 DeRose et al., 2020; Farkasovsky et al., 2005; Garcia et al., 2011; Huijbregts et al., 2009;
87 Iv et al., 2021; John et al., 2007; Kinoshita et al., 2002; Kumagai et al., 2019; Mavrakis et
88 al., 2014; Mendonca et al., 2019; Rosa et al., 2020; Sala et al., 2016; Sirajuddin et al.,
89 2007; Soroor et al., 2021; Versele and Thorner, 2004). Human septins are classified in
90 four homology groups, namely the SEPT2 group (SEPT1, 2, 4, and 5), SEPT6 group
91 (SEPT6, 8, 10, 11, and 14), SEPT7 group (SEPT7), and SEPT3 group (SEPT3, 9, and
92 12) (Kinoshita, 2003). Cell-isolated human septins exist as stable hexamers and octamers
93 (Kim et al., 2011; Sellin et al., 2011; Sellin et al., 2014), with hexamers composed of
94 septins from the SEPT2, SEPT6, SEPT7 groups, and octamers containing additional
95 septins from the SEPT3 group (Fig. 1A).

96 The most convincing evidence that septins form filaments *in vivo* and thereby
97 contribute to cell viability comes from electron microscopy and functional data in budding
98 yeast (Bertin et al., 2012; Byers and Goetsch, 1976; McMurray et al., 2011; Ong et al.,
99 2014; Rodal et al., 2005). The conservation of septins and the ability of mammalian septin
100 hexamers and octamers (hereafter referred to as protomers) to form filaments in solution
101 and on lipid membranes (DeRose et al., 2020; Iv et al., 2021; Leonardo et al., 2021;
102 Soroor et al., 2021; Szuba et al., 2021) has led to the assumption that human septins also
103 organize as filaments in cells, but formal evidence for this is scarce. Immunogold electron
104 microscopy has shown septins closely apposed to cortical actin filaments and to the
105 plasma membrane organizing in linear arrays (Hagiwara et al., 2011; Kinoshita et al.,
106 1997). Septin protomers along actin filaments or the membrane would, however, result in
107 a similar pattern. It is reasonable to assume that septin rings and fiber-looking segments
108 that form in the cytoplasm of mammalian cells upon actin depolymerization correspond
109 to septin filaments or bundles thereof (Joo et al., 2007; Kim et al., 2011; Kinoshita et al.,
110 2002; Schmidt and Nichols, 2004). However, it is unknown if these fibers originate from
111 direct end-to-end septin polymerization. Whether all septins in cells function as filaments,
112 and how hexamers and octamers contribute to septin filament formation and function is
113 not known.

114 Actin-binding domains on septins have not yet been identified. It is hence unclear
115 if actin-septin binding involves direct interactions or if it occurs through myosin-II (Joo et
116 al., 2007; Mostowy et al., 2010) or Borg proteins (Calvo et al., 2015; Farrugia et al., 2020;
117 Joberty et al., 2001; Liu et al., 2014; Salameh et al., 2021). Similarly, although mammalian
118 septins bind lipid membranes (Bridges et al., 2016; Dolat and Spiliotis, 2016; Szuba et
119 al., 2021; Tanaka-Takiguchi et al., 2009; Yamada et al., 2016), whether there is direct
120 septin-membrane binding in cells has not been shown; the identification of the membrane-
121 binding site of septins is a matter of debate (Cavini et al., 2021). It is thus not known if
122 septin-decorated actin fibers and membranes in cells reflect membrane-bound septins.

123 To elucidate the interplay between human septin organization and function in non-
124 dividing cells, we used actin stress fibers in U2OS cells as a model system. Septins in
125 mammalian cells have been reported to decorate stress fibers in multiple studies (Calvo
126 et al., 2015; Connolly et al., 2011; Dolat et al., 2014; Joo et al., 2007; Kim et al., 2011;

127 Kinoshita et al., 2002; Kinoshita et al., 1997; Liu et al., 2014; Salameh et al., 2021;
128 Schmidt and Nichols, 2004; Surka et al., 2002; Verdier-Pinard et al., 2017; Xie et al.,
129 1999; Zhang et al., 1999). Subsets of stress fibers are lost upon septin disruption or septin
130 relocalization to microtubules (Calvo et al., 2015; Kinoshita et al., 2002; Kuzmic et al.,
131 2022; Salameh et al., 2021; Schmidt and Nichols, 2004; Targa et al., 2019) suggesting
132 an essential, yet still unclear, role of septins in stress fiber integrity. To test how septins
133 organize in cells, we combined a tripartite split-GFP complementation assay with mutants
134 disrupting specific septin-septin interfaces to selectively perturb hexamers or octamers,
135 or abolish polymerization altogether. Atomic force microscopy nanoindentation was used
136 to assess the specific contribution of hexamers vs. octamers to cell stiffness. We
137 employed super-resolution structured illumination microscopy to decipher the higher-
138 order assembly of septin filaments. Moreover, to determine if septin filaments are
139 membrane-bound and if they can bridge membrane-actin interactions, we combined
140 nanometer-resolved distance measurements and single protein tracking in cells with cell-
141 free reconstitution assays using supported lipid bilayers. Our findings demonstrate that
142 all actin-associated septins in cells organize as paired membrane-bound filaments whose
143 integrity and function depend on octamers.

144

145 **Results**

146 **Septins associate with contractile stress fibers.** Whether septins associate
147 preferentially with specific types of SFs and if septin organization differs among SFs is
148 not known. To answer these questions, we examined how septins distribute in U2OS
149 cells, with respect to peripheral, dorsal and ventral SFs, transverse arcs and the
150 perinuclear actin cap (Fig. 1A). Given that SFs are classified based on their subcellular
151 localization and their anchoring at one or both ends by focal adhesions (FAs) (Tojkander
152 et al., 2012), we co-stained for septins, actin filaments and the FA protein, paxillin. We
153 examined the distribution of SEPT2 and SEPT7, which are common to both hexamers
154 and octamers, and SEPT9, which is specific to octamers (Fig. 1A,B and Fig. S1). U2OS
155 cells express two SEPT9 isoforms, SEPT9_i1 and SEPT9_i3 (Kuzmic et al., 2022), both
156 of which are detected by our SEPT9 antibodies. Both septin immunostainings and live
157 imaging of septin-GFP fusions showed identical distributions of all three septins with
158 respect to SFs. They all decorated myosin-II containing contractile SFs (Fig. 1Bi-iii; Fig.
159 S1Ai-ii, iv; Fig. S1Bi-v), but not the non-contractile dorsal ones (Fig. 1Biii,b; Fig. S1Aiii;
160 Fig. S1Biv,a). Although septins decorated contractile SFs throughout their length, they
161 were systematically excluded from FAs (Fig. 1Bi,a; Fig. S1Ai,c; Fig. S1Bi,c). Septins
162 localized to peripheral and ventral SFs, transverse arcs and perinuclear actin caps, and
163 also associated with geodesic actin nodes on the ventral plasma membrane and with
164 actin nodes in transverse arcs. Actin nodes were enriched in F-actin and α -actinin, while
165 actin filaments interconnecting nodes were decorated by septins and myosin-II in an
166 aster-like pattern (Fig. S1C,D).

167 The presence of SEPT9 on SFs does not exclude that septin hexamers are also
168 present. Furthermore, the diffraction-limited optical resolution of our setup cannot
169 distinguish septin protomers from filaments. Septin decoration of SFs may therefore
170 reflect the presence of protomers (hexamers and/or octamers) or/and of filaments (with
171 hexamers and octamers forming separate or hybrid filaments) (Fig. 1A).

172
173 **Septins organize as filaments on contractile SFs.** Both protomers have an exposed
174 SEPT2 NC interface at their termini (Fig. 1A) (lv et al., 2021; Mendonca et al., 2019;
175 Soroor et al., 2021). To determine if septins are present as filaments, we therefore
176 designed a tripartite split-GFP complementation assay for probing SEPT2-SEPT2
177 interactions as a molecular readout of end-to-end septin polymerization *in situ* in living
178 cells (Fig. 1C). This protein-protein interaction assay involves the fusion of the proteins of
179 interest to the two last beta-strands of GFP, β 10 and β 11: in the presence of specific
180 protein-protein interactions in cells expressing GFP1-9 (GFP strands β 1- β 9), the GFP
181 barrel is reconstituted leading to fluorescence (Cabantous et al., 2013) (see methods and
182 Fig. S2A-C for the assay design). We generated β 10- and β 11-strand fusions with SEPT2
183 that we co-expressed using an inducible bidirectional vector in U2OS cells constitutively
184 expressing GFP1-9 (Fig. 1C; Fig. S2D). To minimize the risk of not detecting SEPT2-
185 SEPT2 interactions due to endogenous untagged SEPT2 and given that the expression
186 levels of SEPT2- β 10/ β 11 fusions were kept low to minimize overexpression artifacts (Fig.
187 S2E), we consistently knocked down endogenous SEPT2 in all subsequent experiments
188 (Fig. S2F).

189 Confocal imaging revealed the presence of the reconstituted GFP (rGFP) on
190 peripheral and ventral SFs, transverse arcs and perinuclear actin caps (Fig. 1D,E), with
191 the rGFP distribution closely resembling endogenous SEPT2 immunostainings and
192 SEPT2-GFP distribution (Fig. S1A). The presence of SEPT2-SEPT2 rGFP on SFs was
193 further detected in fixed wild-type U2OS cells co-expressing SEPT2- β 10 and SEPT2- β 11
194 upon incubation with recombinant purified GFP1-9 (Fig. 1F), confirming the presence of
195 SEPT2-SEPT2 interactions *in situ* on SFs. Given that recombinant animal septins form
196 both single and paired filaments (Szuba et al., 2021), we generated structure models to
197 examine GFP complementation both from direct SEPT2-SEPT2 interactions within a
198 filament (Fig. 2A), and from SEPT2 facing another SEPT2 in apposed protomers of a
199 paired filament (Fig. 2B). Examination of the distances and the flexibility of the SEPT2 C-
200 termini and the linkers showed that GFP reconstitution could occur either way.

201 The structural models highlighted that paired protomers would lead to GFP
202 reconstitution whether they polymerize or not. Hence to test if SEPT2-SEPT2 rGFP
203 originates from direct SEPT2-SEPT2 interactions, we designed a double point SEPT2 NC
204 interface mutant (SEPT2 F20D, V27D, hereafter SEPT2NCmut) to prevent end-to-end
205 association and thereby abolish polymerization (Fig. S3A) (Kuzmic et al., 2022; Sirajuddin
206 et al., 2007). Reconstitution assays using purified recombinant protomers bearing these
207 mutations confirmed that this mutant abolishes polymerization, although it can still bind

208 actin filaments *in vitro* (Fig. 2C,D and Fig. S3B,C). Native PAGE in cell lysates expressing
209 SEPT2NCmut confirmed that protomers were intact: the expression of either wild-type
210 SEPT2 or SEPT2NCmut in SEPT2 knockdown cells rescues equally well the hexamer
211 and octamer distribution in control cells (Fig. 2E). Strikingly, using this mutant in the
212 context of the split SEPT2-SEPT2 assay completely abolished SF localization, as
213 indicated by purely diffuse cytosolic fluorescence (Fig. 2F,G) (hereafter referred to as
214 "diffuse cytosolic"). Given that wild-type SEPT2-SEPT2 rGFP was occasionally found as
215 diffuse cytosolic, we quantified the distribution of diffuse cytosolic and non-diffuse
216 phenotypes in cells expressing wild-type SEPT2- vs SEPT2NCmut- β 10/ β 11 fusions.
217 While 95% of wild-type SEPT2-SEPT2 rGFP localized to SFs and only 5% was diffuse
218 cytosolic, 100% of SEPT2NCmut-SEPT2NCmut rGFP was diffuse cytosolic (Fig. 2F,G).
219 Thus direct end-to-end septin polymerization is required for septin localization to SFs. We
220 attribute the fact that the split-GFP assay with the NC mutant still produced fluorescence
221 to the plasticity of septins, which are able to use both NC and G interfaces when either
222 one is compromised (Kim et al., 2012). Our native PAGE shows the presence of
223 SEPT2NC homodimers (Fig. 2E) which we speculate are G-homodimers, thus enabling
224 GFP complementation.

225

226 **Septin protomers do not associate with SFs.** The presence of septin filaments does
227 not exclude that septin protomers are also present on SFs. To test if septin protomers
228 associate with SFs, we examined the cellular distribution of SEPT2NCmut fused to full-
229 length GFP. Cells expressing this mutant exhibited a diffuse cytosolic localization,
230 demonstrating that this mutant does not bind SFs (Fig. 2H,I). Wild-type SEPT2-GFP
231 fusions also showed diffuse cytosolic localization in addition to SF localization (Fig.
232 S1A,v). However, while SEPT2-GFP was diffuse cytosolic in only ~50% of cells, 100% of
233 the cells expressing SEPT2NCmut showed this phenotype (Fig. 2H,I). This result showed
234 that septin protomers in cells do not associate with SFs, meaning that all septins
235 decorating SFs are filamentous.

236

237 **SF-associated septin filaments contain predominantly octamers.** As SEPT2 is
238 common to both protomers (Fig. 1A), the results described above did not inform us on the
239 composition of septin filaments. Recombinant hexamers and SEPT9-containing octamers
240 have the capacity to co-polymerize *in vitro* (Fig. S3D and (Soroor et al., 2021)). To
241 explicitly visualize the presence of octamers on SFs, we probed SEPT7-SEPT9 and
242 SEPT9-SEPT9 interactions as molecular signatures of octamers (Fig. 3A,B). Expression
243 levels of all β 10/ β 11 fusions were kept low to minimize overexpression artifacts (Fig. S2E),
244 and endogenous SEPT7 and SEPT9 were consistently knocked down in all subsequent
245 experiments (Fig. S2G,H).

246 As expected, rGFP from SEPT9_{i3}-SEPT9_{i3} localized to contractile SFs (Fig.
247 3C), similarly to SEPT9 immunostainings (Fig. S1B), confirming that septin filaments
248 contain SEPT9_{i3}-octamers. Split-GFP assays probing SEPT7-SEPT9_{i3} interactions

249 confirmed these findings (Fig. 3D), with rGFP additionally labeling cytoplasmic rings of
250 ~0.9 μm in diameter (Fig. 3Dii, E). To confirm that SF-localized rGFP from SEPT7-
251 SEPT9_{i3} and SEPT9_{i3}-SEPT9_{i3} reflect direct interactions, we designed a double
252 point SEPT9_{i3} NC interface mutant (SEPT9_{i3} M263D, I270D, hereafter
253 SEPT9_{i3}NCmut) , a double point SEPT9_{i3} G interface mutant (SEPT9_{i3} W502A,
254 H512D, hereafter SEPT9_{i3}Gmut) and a double point SEPT7 G interface mutant (SEPT7
255 W269A, H279D, hereafter SEPT7Gmut1) (Fig. S3A) (Kuzmic et al., 2022; Sirajuddin et
256 al., 2007; Zent et al., 2011). Native PAGE in cells expressing these mutants confirmed
257 that SEPT9_{i3}NCmut completely disrupts octamers (Fig. 4A), whereas SEPT7Gmut1
258 completely disrupts octamers and hexamers (Fig. 4B). Split-GFP assays using these
259 mutants completely abolished SF localization (Fig. 4C-H), confirming that SF localization
260 requires intact SEPT7-SEPT9_{i3} and SEPT9_{i3}-SEPT9_{i3} interfaces. All above assays
261 gave identical results for SEPT9_{i1}, confirming the presence of both SEPT9_{i1}- and
262 SEPT9_{i3}-containing octamers in SF-associated septin filaments (Fig. 3F, Fig. 4A,H; Fig.
263 S4A,B).

264 To test if hexamers are also present on SFs, we probed SEPT7-SEPT7
265 interactions. Strikingly, rGFP from SEPT7-SEPT7 interactions was unexpectedly difficult
266 to detect on SFs: although it localized to SFs (Fig. 5Ai, Bi), the majority was found on
267 ectopic short, needle-like bundles (Fig. 5Aii, Bii), similar to the localization of full-length
268 GFP-SEPT7 fusions (Fig. 5C,D). These ectopic bundles did not localize to SFs (Fig. 5Aii,
269 D) and contained SEPT2 but not SEPT9 (Fig. 5C). These bundles thus most likely consist
270 of hexamers, in line with the capacity of recombinant hexamers to form septin filament
271 bundles *in vitro* (DeRose et al., 2020; Iv et al., 2021; Kinoshita et al., 2002; Leonardo et
272 al., 2021). The presence of rGFP on the ectopic bundles thus showed that the split
273 SEPT7-SEPT7 assay readily detects SEPT7-SEPT7 interactions originating from
274 hexamers.

275 An observation that could explain the difficulty to detect SEPT7-SEPT7 on SFs
276 was the dependence of SEPT7 localization on SEPT9 expression levels. We consistently
277 detected ectopic bundles when we exogenously expressed only SEPT7, either GFP-
278 SEPT7 or split SEPT7-SEPT7 (Fig. 5A-D), but not when we co-expressed SEPT9 (Fig.
279 5E,F). We reasoned that in the absence of exogenous SEPT9, the slightest excess of
280 SEPT7 leads to ectopic hexamer-based bundles, also reducing the availability of SEPT7
281 for forming octamers to bind SFs. Exogenous co-expression of SEPT9, on the other hand,
282 would cause incorporation of the exogenous SEPT7 into octamers, thus preventing the
283 formation of ectopic hexamer bundles. Consistent with this hypothesis, SEPT7-SEPT7
284 rGFP was readily detectable on SFs under conditions of exogenous SEPT9 co-
285 expression (Fig. 5F). Furthermore, it was difficult to find SEPT9-decorated SFs in cells
286 also displaying ectopic hexamer-based bundles (Fig. 5C). These observations raised the
287 possibility that septin filaments on SFs contain mostly, if not exclusively, octamers.

288 To explore the origin of SEPT7-SEPT7 rGFP on SFs, we generated structure
289 models of septin protomers in order to examine GFP complementation from SEPT7-

290 SEPT7 interactions within one hexamer (Fig. 5G), as well as from SEPT7 facing another
291 SEPT7 in apposed hexamers or octamers in a paired filament (Fig. 5H). Examination of
292 the distances and the flexibility of the SEPT7 N-termini and the linkers showed that GFP
293 reconstitution can occur either way.

294 To identify the sources of the SEPT7-SEPT7 rGFP signal on SFs, we aimed at
295 perturbing hexamers while preserving octamers. To this end, we generated a single point
296 SEPT7 G interface mutant (SEPT7 H279D, hereafter SEPT7Gmut2) (Fig. S3A) that
297 should destabilize the SEPT7-SEPT7 G-interface when present in both SEPT7 subunits,
298 but preserve the SEPT7-SEPT9 G-interface if SEPT7 is mutated but SEPT9 is wild-type.
299 In line with these predictions, native PAGE showed that octamers are not affected by the
300 expression of SEPT7Gmut2 (Fig. 4B), and rGFP from SEPT7Gmut2-SEPT9_i3
301 recapitulated normal septin localization on SFs (Fig. 6A). Importantly, rGFP from
302 SEPT7Gmut2-SEPT7Gmut2 localized to SFs but did not show any ectopic bundles (Fig.
303 6B) indicating that SEPT7Gmut2 completely abolished SEPT7-SEPT7 interactions within
304 hexamers in the bundles. Thus our findings show that the SF-localized rGFP from
305 SEPT7Gmut2-SEPT7Gmut2 originates from paired octamers (Fig. 5H).

306 These observations altogether strongly suggest that the detected rGFP from
307 SEPT7-SEPT7 on SFs originates from paired octamers. Split-GFP assays using the
308 SEPT7Gmut1 mutant resulted in diffuse cytosolic distributions (Fig. 4D,H; Fig. 6C-E),
309 confirming that SF localization requires intact SEPT7 G interfaces. We conclude that
310 septins on SFs organize as paired filaments containing mostly, or even exclusively,
311 octamers.

312

313 **Polymerization-competent septin octamers, but not hexamers, are essential for the**
314 **integrity and function of SF-associated septin filaments.** To further test the
315 contribution of hexamers vs. octamers to septin filament formation, we examined septin
316 filaments under three conditions: (a) the presence of hexamers and octamers (control
317 condition), (b) the absence of octamers, by knocking down SEPT9, and (c) the presence
318 of octamers only, by expressing SEPT7Gmut2. As a readout of septin filaments, we
319 examined rGFP from SEPT2-SEPT2 in live cells while imaging stress fibers (Fig. 7A-C).
320 To assess the effects of the perturbations, we quantified the distribution of non-diffuse vs.
321 diffuse cytosolic phenotypes and calculated Pearson and Manders correlation coefficients
322 for actin-septin co-localization (Fig. 7D,E). Strikingly, removing octamers by knocking
323 down SEPT9 entirely removed the SEPT2-SEPT2 rGFP signal from all SFs, leaving
324 behind a punctate pattern not localizing to SFs, suggesting that filamentous septin
325 integrity depends entirely on octamers. On the other hand, preserving octamers in the
326 absence of hexamers preserved septin filaments on SFs, showing that the absence of
327 hexamers does not compromise septin filament integrity.

328 To test the functional contribution of octamers and hexamers to SFs, we quantified
329 the number of cells presenting ventral SFs in cells containing hexamers and octamers,
330 hexamers only, octamers only, and containing hexamers and octamers that cannot

331 polymerize. Knocking down SEPT9 removed both SEPT9 and SEPT7 from all ventral
332 SFs (compare Fig. 8A and B). Importantly, whereas 82% of control cells, and 81% of cells
333 with octamers only, contained ventral SFs, only 44% of cells in the absence of octamers,
334 and 46% of cells with hexamers and octamers that could not polymerize, presented
335 ventral SFs (Fig. 8B,C). Total actin levels did not change among these conditions (Fig.
336 8D). Thus polymerization-competent septin octamers are essential for the integrity of SFs.

337 To further question the functional contribution of protomers, we turned to atomic
338 force microscopy (AFM) nanoindentation for measuring cell stiffness. Septin depletion
339 has been shown to reduce cell stiffness, using AFM, in cultured mammalian cells
340 (Mostowy et al., 2011), but the specific contribution of hexamers vs octamers was not
341 explored. To address this question, we indented cells containing hexamers and octamers,
342 hexamers only and octamers only with an AFM cantilever tip and determined the elastic
343 modulus (E_0) and the fluidity (β) of the cells by fitting the experimental force-indentation
344 curves to a viscoelastic model (Fig. 8E, see methods). While removing hexamers did not
345 have any effect, the depletion of octamers resulted in a statistically significant decrease
346 in cell stiffness and a corresponding increase in cell fluidity (Fig. 8E). Cells with
347 significantly reduced ventral SFs in the absence of octamers are predicted to generate
348 less prestress and thus lower stiffness (Chowdhury et al., 2021; Wang et al., 2002). We
349 conclude that polymerization-competent octamers are essential for their function in cell
350 mechanics through the generation or/and maintenance of SFs.

351
352 **Super-resolution microscopy reveals septin fibers running longitudinally along**
353 **and around SFs and interconnecting SFs.** Having shown that all septins associated
354 with SFs are filamentous, we aimed at visualizing how septin filaments organize on the
355 different types of SFs. We employed super-resolution structured illumination (SIM)
356 microscopy in cells co-stained for SEPT7 (as a pan-septin filament marker), actin
357 filaments, and α -actinin or non-muscle myosin heavy chain IIA (NMIIA). We examined
358 septin filament organization on perinuclear actin caps, transverse arcs, including at arc
359 nodes, on ventral SFs and at ventral actin nodes (Fig. 9A-E). Regardless of the type of
360 SF, we noticed that septin filament morphology was very different from that of the
361 corresponding actin filament bundles. While actin filament bundles typically appeared as
362 straight, rigid fibers, septin fibers consistently appeared less straight and with lower
363 orientational persistence (*Note: we choose to use septin "fibers" instead of "filaments" in*
364 *this section to avoid confusion with single or paired septin filaments or bundles thereof;*
365 *we discuss the composition of septin fibers below*). Unlike core SF components like
366 myosin and α -actinin, which displayed a sarcomere-like punctate distribution (Fig. 9Ei),
367 septin fibers were distinctly separate from SFs, organizing in three manners: (a) septin
368 fibers running longitudinally along SFs, either on the side of SFs with their signal
369 segregated from the F-actin signal, or overlapping with SFs with the septin and F-actin
370 signals merging (Fig. 9Aa; Ei; Eiii,a,b), (b) septin fibers running longitudinally along SFs
371 and diagonally across their width, as if wrapping around the SFs (Fig. 9Ab; Eii,a,b), and

372 (c) septin fibers running longitudinally along segments of SFs while interconnecting
373 different SFs and connecting to other septin fibers (Fig. 9C; D; Eiv,b). Interconnecting
374 septin fiber segments in between SFs frequently colocalized with F-actin signal, but in
375 many instances there was no detectable F-actin signal along these segments.

376 Regardless of the type of SFs, the majority of septin fibers appeared thinner than
377 their associated SFs. Septin fibers were often thicker on the SF segments adjacent to
378 FAs (Fig. 9Eiii,b; Eiv,a), but thicker septin fibers were also found on arcs, caps and ventral
379 SFs. Thicker septin fibers did not exceed the width of the associated SF, and appeared
380 either as single thick fibers, or what looked like two closely-apposed thin fibers (dashed
381 rectangle in the SEPT7 channel of Fig. 9Eiii). To compare septin fiber thicknesses across
382 the different SF types we measured the width of septin fibers for each SF type. The full
383 width at half maximum (FWHM) was calculated from fluorescence intensity line profiles
384 (Fig. 9F). Widths of the thick septin fibers found on ventral SFs were plotted separately.
385 All thin septin fiber populations had median FWHM values in a narrow range of 123-137
386 nm, whereas the thick ones showed an almost 2-fold higher median FWHM value of 231
387 nm (Fig. 9F,H). There was no statistically significant difference between thin septin fiber
388 widths on caps, asters, arcs and ventral SFs (Fig. 9F).

389 To determine if septin fibers are single or paired filaments ("double septin
390 filaments"), or bundles thereof, we checked the FWHM values of microtubules (MTs) in
391 the same cells. MTs are 25-nm wide tubes and are routinely used as the gold standard
392 for assessing the performance of super-resolution microscopy techniques. MTs were
393 stained using whole primary and fluorophore-coupled secondary IgG antibodies, just like
394 for septin stainings, leading to an estimated real MT width of ~60 nm (Fig. 9I) (Weber et
395 al., 1978). We found an average FWHM value of 115 nm for the MT width in our cells, in
396 line with reported values (Hamel et al., 2014; Wegel et al., 2016), given that the lateral
397 resolution of SIM is roughly half of the diffraction limit, i.e., ~110 nm. Given that the
398 observed size in our images is the convolution of the real object size with the point spread
399 function (PSF) of the SIM microscope, we simulated the predicted image size as a
400 function of the real fiber size (Fig. 9G). The comparison of the estimated real widths of
401 primary and secondary IgG-decorated septins, assumed to organize as single or as
402 paired filaments with either narrow (~5 nm) or wide (~20 nm) spacing (Leonardo et al.,
403 2021) (Fig. 9I), with the widths predicted from our FWHM measurements of
404 immunostained septins (Fig. 9H), suggests that the thin septin fiber widths are compatible
405 with single or paired septin filaments, whereas the thick septin fibers could correspond to
406 two single or two double septin filaments. We note that these estimations assume that
407 septin filaments in cells are present as single filaments with flexible coiled-coils, or paired
408 filaments mediated by coiled-coil pairing (Fig. 9I) in line with published literature (Cavini
409 et al., 2021). The flexibility of coiled-coils combined with the presence of primary and
410 secondary antibodies suggests that septin fibers cannot correspond to more than a few
411 filaments even if septins interact directly through their GTP-binding domains, a condition

412 previously observed only in the absence of coiled-coils (Bertin et al., 2010; Szuba et al.,
413 2021).

414 We also wondered about the length of the SF-associated septin fibers. Short septin
415 fibers associated with and interconnecting actin nodes had lengths on the order of 0.5-
416 3.5 μm , whereas septin fibers on arcs, actin caps and ventral SFs were as short as 0.5-1
417 μm and as long as 10-15 μm (Fig. 9J). We note that these values provide an upper bound
418 since, given the lateral resolution limit of SIM, we cannot be sure that what appears as
419 continuous fiber signal originates from a single fiber or from adjacent fibers overlapping
420 at their ends.

421
422 **All microtubule-associated septins organize as filaments containing**
423 **predominantly octamers.** To examine if our results on SF-associated septin filaments
424 also hold for MT-associated septins, which contain specifically SEPT9_i1 (Kuzmic et al.,
425 2022), we probed SEPT9_i1-SEPT9_i1, SEPT7-SEPT9_i1, and SEPT2-SEPT2
426 interactions using the split-GFP assay. Our results confirmed that MT localization requires
427 intact SEPT7-SEPT9_i1 and SEPT9_i1-SEPT9_i1 interfaces and direct SEPT2-SEPT2
428 interactions (Fig. S4A-F and Fig. 4H), allowing us also to visualize septin filaments on
429 MTs *in situ* in cells (Fig. S4F). We further tested the importance of hexamers for septin-
430 MT association. In line with our results on exogenously expressed SEPT7, it was difficult
431 to find SEPT9-decorated MTs in cells also displaying ectopic hexamer-based bundles
432 (Fig. S4G). rGFP from SEPT7-SEPT7 was, however, readily detected on MTs upon
433 exogenous SEPT9_i1 co-expression (Fig. S4H), suggesting that the rGFP signal may
434 originate from paired filaments (Fig. 5H). rGFP from SEPT7Gmut2-SEPT7Gmut2, in the
435 presence of exogenous SEPT9_i1, and SEPT7Gmut2-SEPT9_i1 were readily detected
436 on MTs (Fig. S4I,J), reflecting SEPT7-SEPT7 rGFP from paired octamers. Finally, SIM
437 imaging revealed thin septin fibers running along MTs over several micrometers (Fig.
438 S4K). Different from the presence of both thin and thick actin-associated septin fibers, all
439 MT-associated septin fibers appeared homogeneous in their width. FWHM
440 measurements (Fig. 9G, Fig. S4L) predicted MT and septin fiber widths in the ranges of
441 65-135 nm and 85-130 nm, respectively, compatible with MT-associated septins
442 organizing as paired septin filaments (Fig. 9I). Our findings reveal that all MT- associated
443 septins are exclusively in the form of octamers and filamentous, consistent with septins
444 on actin SFs.

445
446 **SF-associated septin filaments are closely apposed to the plasma membrane.**
447 Having shown that all SF-associated septins are filamentous, we wondered how septin
448 function relates to septins being filamentous. Recombinant human septins can bind and
449 cross-link actin filaments, but can also bind lipid membranes, raising the hypothesis that
450 septin filaments in cells anchor SFs to the plasma membrane. A first indication that
451 septins might be membrane-bound came from live cell extraction experiments. While
452 extracting the plasma membrane after fixation entirely preserved septin localization to

453 SFs, live-cell extraction removed septins from all SFs while preserving myosin on SFs
454 (Fig. 10A). Septins thus did not behave like core components of SFs, and their sensitivity
455 to the detergent suggested they might be bound to the membrane.

456 To test if septins on SFs are close to the plasma membrane of the U2OS cells, we
457 employed a metal-induced energy transfer (MIET) assay (Chizhik et al., 2014). In MIET,
458 the fluorescence lifetime is dependent on the distance of fluorophores from a metal layer,
459 allowing us to deduce the axial distance of fluorophores from a gold-coated coverslip
460 surface with an axial resolution of a few nanometers (Fig. 10B). We hypothesized that
461 septins could either associate with the plasma membrane while interacting with SFs, or
462 that septins interact with SFs in the absence of any septin-membrane association. To
463 distinguish these scenarios, we compared distances of the fluorescent protein, mApple,
464 in three conditions: (a) mApple N-terminally fused to the 20 N-terminal residues of
465 neuromodulin/GAP43 that contains palmitoylated cysteines (GAP43-mApple); as a
466 reference for fluorophores localizing directly at the plasma membrane, (b) SEPT9_i3-
467 mApple as a reference for ventral SF-associated septin octamers, and (c) SEPT9_i3-
468 mApple-CAAX as a reference for septins targeted to the plasma membrane through the
469 H-Ras CAAX motif which functions as a membrane targeting signal. Representative
470 lifetime decay traces are shown in Fig. 10C and Fig. S5A,B. Strikingly, the distance of
471 mApple from the metal surface, derived from the lifetime-distance dependence curve (Fig.
472 10D), was the same for SF-associated septins, membrane-bound mApple, and
473 membrane-bound septins, meaning that septins are closely apposed to the plasma
474 membrane (Fig. 10E). Lifetime measurements of AF568-phalloidin bound to ventral SFs
475 under the same conditions placed SFs significantly further away, by ~25 nm, from the
476 plasma membrane (Fig. 10E). MIET assays being limited to probing interactions within
477 200 nm from the metal surface, it was not feasible to probe septin populations on
478 transverse arcs and perinuclear actin caps, which are localized further away.

479
480 **Septin filaments anchor actin filaments to lipid membranes.** Since SF-associated
481 septins are closely apposed to the membrane, we wondered if septin filaments could
482 function to anchor stress fibers to the plasma membrane. In the absence of available
483 septin membrane-binding and actin-binding mutants, we turned to reconstitution assays
484 on supported lipid bilayers (SLBs), comparing only phosphatidylcholine- (PC) vs
485 phosphatidylinositol(4,5)-bisphosphate (PI(4,5)P₂)-containing membranes (Fig. 10F),
486 PI(4,5)P₂ being a septin-interacting lipid (Szuba et al., 2021). To image only truly
487 membrane-associated structures, we used total internal reflection fluorescence (TIRF)
488 microscopy in the absence of crowding agents. Actin filaments alone did not bind lipid
489 membranes, whereas septin octamers alone specifically bound PI(4,5)P₂-containing
490 membranes (Fig. S5C,D), in line with previous reports for mammalian septin hexamers
491 (Szuba et al., 2021). To test if septins can anchor actin to membranes, we either
492 preformed actin-septin bundles in solution and then added them to SLBs, co-polymerized
493 septins and actin on SLBs, or added actin to preassembled septin filaments on SLBs. In

494 all cases, and specifically on PI(4,5)P₂-containing membranes but not on membranes
495 composed only of PC, actin filaments and actin filament bundles were anchored to the
496 lipid bilayers (Fig. 10F; Fig. S5E,F), showing that septin filaments can indeed at the same
497 time bind membranes and actin and thus mediate membrane-actin anchoring.

498

499 **Single protein tracking reveals that septins are immobilized on actin stress fibers.**

500 To determine if the molecular dynamics of septins at the plasma membrane are consistent
501 with a SF-anchoring function, we combined photoactivated localization microscopy
502 (PALM) with live-cell single protein tracking of SEPT9_i3 and actin fused to
503 photoswitchable mEos fluorescent proteins using sptPALM (Manley et al., 2008; Rossier
504 et al., 2012). Cells were co-transfected with mEos-fused proteins and EYFP-paxillin as a
505 FA reporter, or GFP-actin as a SF and FA reporter. Using TIRF microscopy, we detect
506 and track sparse photo-activated proteins within 200 nm above the coverslip surface at
507 high-frequency (50Hz acquisition), allowing us to reconstruct thousands of protein
508 trajectories (Fig. 10G,H,I). For trajectories lasting at least 260 ms, we compute the mean
509 square displacement (MSD). We then sort trajectories according to their diffusion modes
510 (immobile, confined, free-diffusive), and extract diffusion coefficients (D_{diff} , D_{conf}) (Fig.
511 10J,K; Fig. S5G,H; see methods). We first looked at the dynamic behavior of mEos2-actin
512 in SFs labelled with GFP-actin (Fig. 10G). mEos2-actin was found inside FAs and also
513 linearly organised along SFs between FAs, as expected. Actin mostly displayed
514 immobilized and confined behaviors, as illustrated by the large fractions of immobilization
515 and confined diffusion (Fig. 10G,K; immobile: $88.5 \pm 0.5\%$, confined: $6.8 \pm 0.3\%$, mean \pm
516 SEM) and a distribution of diffusion coefficients centered around $1.5\text{-}2.5 \cdot 10^{-3} \mu\text{m}^2 \cdot \text{s}^{-1}$ (Fig.
517 10J). In line with septin immunostainings, single SEPT9_i3-mEos3.2 molecules were
518 rarely found inside FAs, but were linearly organized between FAs decorating SFs (Fig.
519 10H). Like actin, also SEPT9_i3-mEos3.2 was found to be primarily immobilized and
520 confined (Fig. 10H,K; immobile: $70.0 \pm 1.9\%$, confined: $13.9 \pm 0.8\%$, mean \pm SEM).
521 Contrary to actin, however, SEPT9_i3-mEos3.2 also displayed a significant freely
522 diffusing population (Fig. 10H,K; diffusive: $16.1 \pm 1.3\%$, mean \pm SEM). However, septin
523 free-diffusion was very slow (Fig. 10J; Fig. S5G) with a diffusion constant $D_{diff} = 0.087 \pm$
524 $0.001 \mu\text{m}^2 \cdot \text{s}^{-1}$ (mean \pm SEM) that is comparable to that of free diffusing transmembrane
525 proteins (integrins: (Rossier et al., 2012)) or of a lipid-anchored protein bound to the
526 plasma membrane by its PH domain (kindlin: (Orre et al., 2021)). Confined SEPT9_i3-
527 mEos3.2 also diffused very slowly (Fig. S5H) with a diffusion constant $D_{conf} = 0.044 \pm$
528 $0.001 \mu\text{m}^2 \cdot \text{s}^{-1}$, comparable to that of mEos2-actin ($0.057 \pm 0.003 \mu\text{m}^2 \cdot \text{s}^{-1}$; means \pm SEM).
529 Overall these results suggest that septins, when immobilized and confined, could indeed
530 be anchoring actin SFs to the plasma membrane, while the free-diffusing septins display
531 a diffusivity that is consistent with them being membrane-anchored.

532 Similarly to the MIET experiments, we used SEPT9_i3-mEos3.2-CAAX as a
533 reference for septins targeted to the plasma membrane. SEPT9_i3-mEos3.2-CAAX did
534 not localize specifically to SFs but decorated the whole plasma membrane (Fig. 10I). In

535 comparison with the behavior of SEPT9_i3-mEos3.2, SEPT9_i3-mEos3.2-CAAX
536 displayed a smaller immobilized fraction (Fig. 10I,J,K; immobile: $32.6 \pm 0.5\%$) but
537 increased free diffusion and confined diffusion fractions with an increased diffusion
538 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.
539 S5G,H). Interestingly, being stably anchored to the plasma membrane allowed
540 SEPT9_i3-mEos3.2-CAAX to diffuse inside FAs (Fig. 10I). The much lower diffusion
541 coefficient of SEPT9_i3-mEos3.2 compared to that of SEPT9_i3-mEos3.2-CAAX is in line
542 with freely diffusing SEPT9_i3 being fully incorporated into septin filaments and could
543 reflect hop diffusion of septin filaments alternating between SFs and the plasma
544 membrane.

545

546 **Septins can associate with actin filaments in cells in a myosin-II independent**
547 **manner.** Although septins decorate myosin-II containing SFs in cells, reconstituted septin
548 filaments can mediate membrane-actin anchoring in the absence of myosin-II. To address
549 if myosin-II is required for binding septins to SFs, we examined the distribution of F-actin,
550 septins and the two most abundant non-muscle myosin heavy chain isoforms IIA (NMIIA)
551 and IIB (NMIIB) in wild-type U2OS cells, in CRISPR-mediated NMIIA and NMIIB knock-
552 out (KO) U2OS lines (Kage et al., 2022), as well as in NMIIA KO cells treated with NMIIB
553 siRNA to remove both isoforms. Both NMIIA and NMIIB were present on septin-decorated
554 SFs in wild-type cells (Fig. S5I i,ii). In NMIIA KO cells, NMIIB still associated with septin-
555 decorated SFs (Fig. S5I iii), and NMIIA still associated with septin-decorated SFs in NMIIB
556 KO cells (Fig. S5I iv). Thus septin association with SFs does not depend specifically on
557 NMIIA or on NMIIB. However, there is 78% identity among NMIIA and NMIIB, including
558 within the coiled-coil region previously implicated in septin binding (Joo et al., 2007),
559 raising the possibility that a shared septin-binding sequence could bind septins to SFs.
560 To assess septin-SF binding in the absence of myosin-II, we turned to NMIIA KO cells
561 treated with NMIIB siRNA (Fig. S5J,K). NMIIA- and NMIIB-depleted cells showed a loss
562 of SFs with dramatic cell shape changes, including "C" and dendritic shapes previously
563 reported upon NMII inhibition or silencing (Cai et al., 2010). Interestingly, F-actin
564 appeared as a dense mesh of loosely cross-linked filaments as well as arrays of asters,
565 with septin filaments associating extensively with actin filaments in the meshes and asters
566 (Fig. S5K). Septins are thus able to associate with actin filaments in cells in a myosin-II
567 independent manner.

568

569 **Discussion**

570 Employing a tripartite split-GFP complementation assay to probe SEPT2-SEPT2
571 interactions as a molecular readout for end-to-end septin polymerization, we showed that
572 all septins on SFs organize as filaments. Mutants that disrupt specifically the SEPT7-
573 SEPT7 interface allowed us to distinguish the contributions of hexamers vs. octamers.
574 Our results showed that septin filaments on SFs contain exclusively octamers, which are
575 essential for the integrity of septin filaments and the integrity of SFs. Septin fiber widths

576 measured by SIM microscopy are compatible with their organization as paired septin
577 filaments. Nanometer-resolved distance measurements and single-protein tracking
578 showed that septin filaments are closely apposed to the plasma membrane and largely
579 immobilized on SFs. Finally, reconstitution assays showed that septin filaments mediate
580 actin-membrane anchoring. We propose that septin filaments anchor and stabilize actin
581 fibers at the plasma membrane (working model in Fig. 10L).

582 Whether septins organize as filaments in a specific cellular context can now be
583 tested using the tools we have developed in this study, including in genetic animal model
584 systems. Our mutants and split-GFP assays are easily adapted for other septins within
585 the SEPT2 group when required by cell- and tissue-specific expression (Karlsson et al.,
586 2021; Uhlen et al., 2015). Split-GFP assays can also be used as readouts for filament
587 formation in the context of studies aiming to identify regulators or inhibitors of septin
588 polymerization, or septin mutants related to disease.

589 An unexpected result of our study is that human septin filament integrity in cells
590 depends entirely on octamers, questioning the functional importance of hexamers. We
591 speculate that it is the SEPT3 group septin, which is absent from hexamers, that dictates
592 septin function. The observation that SEPT7 assembly is most sensitive to SEPT7 and
593 SEPT9 expression levels, and the fact that SEPT7-SEPT7 interactions are stronger than
594 SEPT7-SEPT9 ones (Rosa et al., 2020) suggest that SEPT9, and possibly the other
595 SEPT3 group septins, may help prevent SEPT7 from forming ectopic bundles. It is
596 intriguing that *Drosophila* does not have any SEPT3 group septins and thus contains only
597 hexamers (Field et al., 1996). Interestingly, *Drosophila* septins occasionally form
598 cytoplasmic bundles devoid of Anillin (Hickson and O'Farrell, 2008). Such bundles also
599 form in the absence of Anillin's septin-binding domain that recruits septins to the plasma
600 membrane (Kechad et al., 2012). We speculate that the formation of these bundles is
601 analogous to the ectopic hexamer bundles we observed, forming at limiting amounts of a
602 physiological partner.

603 Our findings suggest that actin-associated septin filaments in mammalian cells
604 organize as paired septin filaments, with thicker septin fibers consisting of 2-3 double
605 septin filaments. One single-molecule localization microscopy study of SF-associated
606 septins has reported septin bundles with 25-150 filaments (Vissa et al., 2019), but this
607 study assumed septin GTP-binding domains associating laterally without considering the
608 presence of coiled-coils and the spacing occupied by coiled-coil pairing (Leonardo et al.,
609 2021), so the number of filaments could be overestimated. We thus hypothesize that
610 human septins in cells, like budding yeast septins, organize in a rather narrow range of
611 assembly geometries. We speculate that the wide range of assembly geometries found
612 for reconstituted septins in solution reflects their plasticity, but that the presence of a
613 physiological partner leads to their native assembly into paired filaments (Bertin et al.,
614 2010; Ong et al., 2014; Szuba et al., 2021).

615 Mammalian septins are distinct from other membrane-bound actin-binding and -
616 crosslinking proteins in that they form filaments. Their capacity to polymerize, catalyzed

617 by membrane binding (Szuba et al., 2021) and coupled with their ability to bind and cross-
618 link actin filaments (Iv et al., 2021; Mavrakis et al., 2014), provides them with the unique
619 potential to stabilize actin filament bundles and meshes at the plasma membrane over
620 considerable distances. We propose that septins function by stabilizing ventral SFs,
621 transverse arcs and perinuclear actin caps at the respective ventral and dorsal plasma
622 membrane, participating in their generation or/and maintenance. It will be interesting to
623 explore if cortical actin meshworks are also membrane-attached via septins (Vadnjaj et
624 al., 2022). Septin enrichment adjacent to FAs lets us propose that they also contribute a
625 stabilization function at the connection between FAs and SFs and thus impact FA
626 maturation indirectly (Calvo et al., 2015; Dolat et al., 2014; Kang et al., 2021) by affecting
627 the accumulation of mechanical tension on SFs.

628 The findings of this study lead to several remaining open questions. Whether
629 septins in cells bind membranes and actin filaments directly or indirectly remains to be
630 shown. Our data show that septins can associate with actin fibers in cells in the absence
631 of myosin-II, supporting the possibility of direct septin-actin interactions in the context of
632 SFs. The fact that septins are found only on contractile SFs suggests that myosin-II
633 related signaling might be involved in their recruitment to SFs. Also what regulates septin
634 polymerization in cells is still unknown. Cell-free reconstitution approaches and animal
635 model systems promise to provide important further insights into the link between animal
636 septin organization and function.

637

638 **Figure legends**

639 **Figure 1. A tripartite split-GFP complementation assay detects SEPT2-SEPT2**
640 **interactions on septin-decorated actin stress fibers in U2OS cells. (A)** The scheme
641 on the left depicts septin-decorated stress fibers (SFs) in a mammalian cell. Septins
642 (green) decorate different types of actin SFs (red), notably peripheral and ventral SFs (i),
643 perinuclear actin caps (ii) and transverse arcs (iii), shown in the respective panels (i-iii) in
644 (B). The schematics on the right show human septin octamers and hexamers associating
645 with SFs either as single protomers (top), as octamer and hexamer-driven filaments
646 (middle), or as hybrid filaments from octamer and hexamer co-polymerization (bottom).
647 Single protomers (top) could co-exist with filaments (middle and bottom). Septins can
648 associate exclusively with SFs or also with the plasma membrane. **(B)** Representative
649 confocal micrographs of SEPT7 immunostainings showing examples of SEPT7 localizing
650 (i) to ventral (a,b) and peripheral (c) SFs and excluded from focal adhesions (FA) (a), (ii)
651 to perinuclear actin caps (a,b), and (iii) to transverse arcs (a) and excluded from dorsal
652 SFs (b). Cells are co-stained for F-actin (phalloidin) and the FA protein paxillin. **(C)**
653 Schematic representation of the tripartite split-GFP complementation assay for probing
654 SEPT2-SEPT2 interactions. The transparency of the SEPT7 subunits is used to suggest
655 that the polymerizing protomers can be hexamers or/and octamers. **(D-E)** Representative
656 confocal micrographs of SEPT2-SEPT2 reconstituted GFP (rGFP) distribution in fixed
657 cells (D) co-stained for F-actin (phalloidin) and in live cells (E). Examples in fixed cells

658 show rGFP localizing (i) to ventral SFs (a,b) and (ii) to actin caps (a,b). Examples in live
659 cells show rGFP localizing (i) to peripheral (a) and ventral (b) SFs, (ii) to transverse arcs
660 (a,b), and (iii) to actin caps (a,b). **(F)** Representative confocal micrograph of SEPT2-
661 SEPT2 rGFP in fixed cells using recombinant purified GFP1-9. The example shows rGFP
662 localizing to ventral SFs (a,b). Scale bars in large fields of views, 10 μm . Scale bars in
663 insets, 2 μm .

664
665 **Figure 2. All septins on SFs organize as filaments. (A-B)** Structure models of rGFP via
666 direct SEPT2-SEPT2 interactions of two polymerizing septin protomers within a filament
667 (A) or from SEPT2 in two apposed protomers (B). Only the end-to-end interacting halves
668 of the protomers (hexamers or/and octamers) are shown in (A) for simplicity. SEPT6 and
669 SEPT7 coiled-coils are not shown in (B) for simplicity. The transparency of the terminal
670 SEPT2 subunits in (B) is used to suggest that the paired protomers could be found within
671 a filament. β 10 and β 11 strands are shown in yellow and orange, respectively. Linker
672 sequences between septins and the β -strands, delimited by arrowheads, are shown in
673 dark grey. The colors of septin subunits in the structure models correspond to the ones
674 in the color-coded sphere representation of hexamers and octamers. The second half of
675 the octamer is not shown in the rotated filament pair in (B) for the sake of simplicity. **(C)**
676 Representative spinning disk fluorescence images of septin filament assembly upon
677 polymerization of octamers-9_i3 in solution at the indicated final protomer concentration.
678 Protomers contained either wild-type SEPT2 (left panel) or SEPT2NCmut (right panel).
679 Images use an inverted grayscale. **(D)** Representative spinning disk fluorescence images
680 of reconstituted actin filaments, polymerizing in the presence of septin octamers in
681 solution. Protomers contained either wild-type SEPT2 (left panel) or SEPT2NCmut (right
682 panel). Actin filaments are visualized with AlexaFluor568-conjugated phalloidin, and
683 septins with SEPT2-msfGFP. One example of large fields of view are shown for each
684 condition, depicting cross-linking of actin filaments; only actin labeling is shown. Insets on
685 the bottom show higher magnifications of selected regions of interest on the top (dashed
686 squares in red). Two regions of interest (a,b for wild-type SEPT2 and c,d for
687 SEPT2NCmut) are shown in each case, depicting both the actin (top row) and septin
688 (bottom row) signals. Scale bars in all large fields of views, 10 μm . Scale bars in all insets,
689 5 μm . **(E)** Western blot following native PAGE of U2OS cell lysates probed with anti-
690 SEPT7 (left) and anti-SEPT2 (right) antibodies upon treatment with siRNAs targeting
691 LacZ (siCtrl), SEPT2 (siSEPT2), and targeting SEPT2 while expressing wild-type SEPT2-
692 msfGFP (WT) or SEPT2NCmut-msfGFP (NCmut). Molecular weight markers are shown
693 on the left. The overexpression of the msfGFP fusions leads to SEPT2 monomers and
694 dimers in addition to hexamers and octamers (arrowheads). **(F)** Violin plots depicting the
695 distribution of diffuse cytosolic (red datapoints) vs. non-diffuse (green datapoints)
696 phenotypes as a function of the intensity of the rGFP signal in GFP1-9 cells co-expressing
697 wild-type SEPT2- β 10 and - β 11 or SEPT2NCmut- β 10 and - β 11. Data points are from a
698 total of 40 cells each for wild-type and mutant SEPT2 distributed among the two

699 phenotypes. **(G)** Representative example of a GFP1-9 cell co-expressing SEPT2NCmut-
700 β 10 and - β 11 and co-stained for F-actin (phalloidin) showing a diffuse cytosolic
701 phenotype. Scale bar, 10 μ m. **(H)** Violin plots depicting the distribution of diffuse cytosolic
702 (red datapoints) vs. non-diffuse (green datapoints) phenotypes as a function of the
703 intensity of the msfGFP signal in cells expressing wild-type SEPT2-msfGFP or
704 SEPT2NCmut-msfGFP. Data points are from a total of 90 cells each for wild-type and
705 mutant SEPT2 distributed among the two phenotypes. **(I)** Representative example of a
706 cell expressing SEPT2NCmut-msfGFP and co-stained for F-actin (phalloidin) showing a
707 diffuse cytosolic phenotype. Scale bar, 10 μ m.

708

709 **Figure 3. SF-associated septin filaments contain octamers. (A-B)** Schematic (top) and
710 respective structure model (bottom) of rGFP via SEPT9-SEPT9 interactions (A) and
711 SEPT7-SEPT9 interactions (B) within an octamer. The transparency of the terminal
712 SEPT2 subunits is used to suggest that the protomers are found within a filament. β 10
713 and β 11 strands are shown in yellow and orange, respectively. Linker sequences between
714 septins and the β -strands, delimited by arrowheads, are shown in dark grey. The colors
715 of septin subunits in the structure models match the ones in the color-coded sphere
716 representation of octamers. **(C)** Representative confocal micrographs of SEPT9_i3-
717 SEPT9_i3 rGFP distribution in fixed cells (left and middle columns) co-stained for F-actin
718 (phalloidin) and in live cells (right column). Examples of rGFP in fixed cells localizing (i)
719 to ventral (a) and peripheral (b) SFs and (ii) to transverse arcs (b) and excluded from
720 dorsal SFs (a). Examples in live cells show rGFP localizing (iii) to ventral SFs (a,b) and
721 (iv) to actin caps (a,b). Scale bars in large fields of views, 10 μ m. Scale bars in insets, 2
722 μ m. **(D)** Representative confocal micrographs of SEPT7-SEPT9_i3 rGFP distribution in
723 fixed cells (left and middle columns) co-stained for F-actin (phalloidin) and in live cells
724 (right column). Example of rGFP in fixed cells localizing (i) to ventral SFs (a,b). Example
725 in live cells showing rGFP localizing (ii) to transverse arcs (a,b). The arrowhead points to
726 a ring. Such cytoplasmic rings were \sim 0.5-1.6 μ m in diameter (0.9 μ m on average from 19
727 measured rings). Scale bars in large fields of views, 10 μ m. Scale bars in insets, 2 μ m.
728 **(E)** Additional examples of rings (arrowheads) in GFP1-9 cells co-expressing β 11-SEPT7
729 and SEPT9_i3- β 10. Scale bar, 2 μ m. **(F)** Representative confocal micrographs of SEPT7-
730 SEPT9_i1 rGFP distribution in fixed cells (left and middle columns) co-stained for F-actin
731 (phalloidin) and in live cells (right column). Examples in fixed and live cells show rGFP
732 localizing to ventral SFs (a,b). Scale bars in large fields of views, 10 μ m. Scale bars in
733 insets, 2 μ m.

734

735 **Figure 4. Intact SEPT9 NC and G interfaces are required for septin localization to**
736 **SFs. (A)** Western blot following native PAGE of U2OS cell lysates probed with anti-
737 SEPT9 (left and middle) and anti-SEPT7 (right) antibodies upon treatment with siRNAs
738 targeting LacZ (siCtrl), SEPT9 (siSEPT9), and targeting SEPT9 while expressing wild-

739 type SEPT9-msfGFP (WT) or SEPT9NCmut-msfGFP (NCmut) for both SEPT9_i1 and
740 SEPT9_i3. The SEPT9 blot is also shown saturated on purpose for displaying weaker
741 bands. Arrowheads point to the sizes of the indicated complexes. The asterisks point to
742 SEPT9 degradation. Molecular weight markers are shown on the left. **(B)** Western blot
743 following native PAGE of U2OS cell lysates probed with anti-SEPT9 (left) and anti-SEPT7
744 (right) antibodies upon treatment with siRNAs targeting LacZ (siCtrl), SEPT7 (siSEPT7),
745 and targeting SEPT7 while expressing wild-type msfGFP-SEPT7 (WT), msfGFP-
746 SEPT7Gmut1 (Gmut1), or msfGFP-SEPT7Gmut2 (Gmut2). Arrowheads point to the sizes
747 of the indicated complexes. Molecular weight markers are shown on the left. **(C)**
748 Representative example of a GFP1-9 cell co-expressing SEPT9_i3NCmut- β 10 and - β 11,
749 co-stained for F-actin (phalloidin), showing a diffuse cytosolic phenotype. Scale bar, 10
750 μ m. **(D)** Representative examples of a fixed (i) and a live (ii) GFP1-9 cell co-expressing
751 β 11-SEPT7Gmut1 and SEPT9_i3Gmut- β 10 showing a diffuse cytosolic phenotype. The
752 fixed cell is co-stained for F-actin (phalloidin). Scale bar, 10 μ m. **(E)** Violin plots depicting
753 the distribution of diffuse cytosolic (red datapoints) vs. non-diffuse (green datapoints)
754 phenotypes as a function of the intensity of the msfGFP signal in cells expressing wild-
755 type SEPT9_i3-msfGFP or SEPT9_i3NCmut-msfGFP. Data points are from a total of 90
756 cells each for wild-type and mutant SEPT9 distributed among the two phenotypes. **(F)**
757 Representative example of a cell expressing SEPT9_i3NCmut-msfGFP, co-stained for F-
758 actin (phalloidin), showing a diffuse cytosolic phenotype. Scale bar, 10 μ m. **(G)** Violin
759 plots depicting the distribution of diffuse cytosolic (red datapoints) vs. non-diffuse (green
760 datapoints) phenotypes as a function of the intensity of the rGFP signal in GFP1-9 cells
761 co-expressing wild-type SEPT9_i3- β 10 and - β 11 or SEPT9_i3NCmut- β 10 and - β 11. Data
762 points are from a total of 40 cells each for wild-type and mutant SEPT9 distributed among
763 the two phenotypes. **(H)** Violin plots depicting the distribution of diffuse cytosolic (red
764 datapoints) vs. non-diffuse (green datapoints) phenotypes as a function of the intensity of
765 the rGFP signal in GFP1-9 cells co-expressing the indicated combinations of β 11-SEPT7
766 and SEPT9- β 10 fusions. Data points are from a total of 40 cells each for each
767 combination, distributed among the two phenotypes.

768
769 **Figure 5. Exogenous SEPT7 and SEPT9 expression affect SEPT7 distribution. (A)**
770 Representative confocal micrographs of SEPT7-SEPT7 rGFP distribution in fixed cells
771 co-stained for F-actin (phalloidin) localizing (i) to ventral (a,b) SFs and (ii) to ectopic
772 bundles devoid of phalloidin staining (a,b). Scale bars in large fields of views, 10 μ m.
773 Scale bars in insets, 2 μ m. **(B)** Representative confocal micrographs of SEPT7-SEPT7
774 rGFP distribution in live cells localizing (i) to transverse arcs (a,b) and (ii) to ectopic
775 bundles (a,b). Scale bars in large fields of views, 10 μ m. Scale bars in insets, 2 μ m. **(C)**
776 Examples of cells expressing msfGFP-SEPT7 and co-stained for SEPT2 (i) or for SEPT9
777 (ii). msfGFP-SEPT7 localizing to ectopic bundles contained SEPT2 (i;a,b) but not SEPT9
778 (ii;a). A non-transfected cell in (ii) shows SEPT9-stained SFs (b). Scale bars in large fields
779 of views, 10 μ m. Scale bars in insets, 2 μ m. **(D)** Representative confocal micrograph of a

780 cell expressing msfGFP-SEPT7 co-stained for F-actin (phalloidin) localizing to ventral
781 SFs (a) and to ectopic bundles devoid of phalloidin staining (b). Scale bars in large fields
782 of views, 10 μm . Scale bars in insets, 2 μm . **(E)** Representative example of a cell (top
783 right) co-expressing msfGFP-SEPT7 and SEPT9_i3-mApple and labeled for F-actin (SiR-
784 actin). Example shows msfGFP-SEPT7 localizing to ventral SFs (b). A cell expressing
785 only msfGFP-SEPT7 (bottom left) in (ii) shows msfGFP-SEPT7 localizing to ectopic
786 bundles that are devoid of F-actin (a). Scale bars in large fields of views, 10 μm . Scale
787 bars in insets, 2 μm . **(F)** Representative examples of GFP1-9 cells co-expressing β 10-
788 and β 11-SEPT7, SEPT9_i3-mApple and labeled for F-actin (SiR-actin). Example in (i)
789 shows rGFP localization to ventral SFs. Example in (ii) shows rGFP localization to
790 peripheral (a), ventral SFs (b) and transverse arcs (c). Scale bars in large fields of views,
791 10 μm . Scale bars in insets, 2 μm . **(G-H)** Schematic (top) and respective structure model
792 (bottom) of rGFP via SEPT7-SEPT7 interactions within a hexamer (G) or from SEPT7 in
793 two apposed octamers within a paired filament (H). The transparency of the terminal
794 SEPT2 subunits is used to suggest that the protomers are found within a filament. β 10
795 and β 11 strands are shown in yellow and orange, respectively. Linker sequences between
796 septins and the β -strands, delimited by arrowheads, are shown in dark grey. The colors
797 of septin subunits correspond to the ones in the color-coded sphere representation of
798 hexamers and octamers. The second half of the octamer is not shown in the rotated
799 filament pair in (H) for the sake of simplicity. Only SEPT7 subunits are shown in the zoom-
800 in of the reconstituted GFP barrel in G for the sake of simplicity.

801
802 **Figure 6. Intact SEPT7 G interfaces are required for septin localization to SFs. (A)**
803 Representative examples of fixed (i,ii) and live (iii-vi) GFP1-9 cells co-expressing β 11-
804 SEPT7Gmut2 and SEPT9_i3- β 10. Fixed cells are co-stained for F-actin (phalloidin).
805 Examples shows rGFP localizing (i,ii) to ventral SFs (a,b), (iii) to perinuclear actin caps
806 (a,b), (iv) to ventral SFs (a,b), and (v-vi) rings (arrowheads). Scale bars in large fields of
807 views, 10 μm . Scale bars in insets, 2 μm . **(B)** Representative examples of GFP1-9 cells
808 (i-iv) co-expressing β 10- and β 11-SEPT7Gmut2. The fixed cell is co-stained for F-actin
809 (phalloidin). Examples show diffuse cytosolic phenotypes (i,iii) of the rGFP and rGFP
810 localizing to SFs (ii, iv). Scale bars in large fields of views, 10 μm . Scale bars in insets, 2
811 μm . **(C)** Violin plots depicting the distribution of diffuse cytosolic (red datapoints) vs. non-
812 diffuse (green datapoints) phenotypes as a function of the intensity of the msfGFP signal
813 in cells expressing wild-type msfGFP-SEPT7 or msfGFP-SEPT7NCmut. Data points are
814 from a total of 71 cells for wild-type, 68 cells for SEPT7Gmut1 and 90 cells for
815 SEPT7Gmut2 distributed among the two phenotypes. **(D)** Violin plots depicting the
816 distribution of diffuse cytosolic (red datapoints) vs. non-diffuse (green datapoints)
817 phenotypes as a function of the intensity of the rGFP signal in GFP1-9 cells co-expressing
818 wild-type β 10- and β 11-SEPT7, β 10- and β 11-SEPT7Gmut1, or β 10- and β 11-
819 SEPT7Gmut2. Data points are from a total of 40 cells for wild-type, 33 cells for β 10- and

820 β 11-SEPT7Gmut1 and 29 cells for β 10- and β 11-SEPT7Gmut2 distributed among the two
821 phenotypes. **(E)** Representative examples of GFP1-9 cells (i,ii) co-expressing β 10- and
822 β 11-SEPT7Gmut1 showing a diffuse cytosolic phenotype. The fixed cell is co-stained for
823 F-actin (phalloidin). Scale bar, 10 μ m.

824

825 **Figure 7. Septin octamers are essential for the integrity of SF-associated septin**
826 **filaments. (A-C)** Representative confocal micrographs of SEPT2-SEPT2 rGFP
827 distribution in live cells co-labeled for F-actin (SiR-actin). Cells were treated with siRNA
828 targeting SEPT2 (A), with siRNAs targeting both SEPT2 and SEPT9 (B), or with siRNA
829 targeting both SEPT2 and SEPT7 and co-transfected with mApple-SEPT7Gmut2 (C).
830 Examples in (A) and (C) show rGFP localizing to ventral SFs (a,b). Scale bars in large
831 fields of views, 10 μ m. Scale bars in insets, 2 μ m. **(D)** Scatter dot plots depicting the
832 distribution of diffuse cytosolic (red datapoints) vs. non-diffuse (green datapoints)
833 phenotypes in cells under the same conditions as in (A-C), also shown as pie graphs.
834 Data points are from a total of 59 cells for wild-type and 60 cells for each perturbation
835 condition, distributed among the two phenotypes. **(E)** Scatter dot plots (mean \pm SD)
836 depicting the distributions of calculated Pearson (left) and Manders (right) correlation
837 coefficients for actin-septin colocalization in cells under the same conditions as in (A-C).
838 Data points for each plot, from left to right, are from a total of 30, 37 and 46 cells,
839 respectively. Kruskal-Wallis test; *** P<0.001, **** P<0.0001.

840

841 **Figure 8. Ventral SFs are significantly reduced in the absence of octamers or upon**
842 **impairing septin polymerization. (A)** Representative confocal micrograph of cells co-
843 stained for SEPT7, SEPT9 and F-actin (phalloidin). Examples show septins localizing to
844 ventral SFs (a,b). **(B)** Representative confocal micrographs of wild type U2OS cells
845 treated with siRNA targeting SEPT9 and co-stained for SEPT7, SEPT9 and F-actin
846 (phalloidin). Example (i) depicts cells with no ventral SFs, whereas example (ii) shows
847 cells that have ventral SFs that are devoid of septins (a,b) (see methods for classification).
848 **(C)** Box plots showing the frequency of ventral SF presence in wild-type cells treated with
849 siRNA targeting LacZ (6mer+8mer), siRNA targeting SEPT9 (6mer), siRNA targeting
850 SEPT7 and co-transfected with msfGFP-SEPT7Gmut2 (8mer), and siRNA targeting
851 SEPT2 and co-transfected with SEPT2NCmut-msfGFP (no filaments). The data points
852 are plotted on top of the respective box plots; each data point corresponds to one round
853 of experiments. On each box, the central mark indicates the median, and the bottom and
854 top edges of the box indicate the 25th and 75th percentiles, respectively. The whiskers
855 extend to the minimum and maximum values. The number of cells analyzed per
856 experiment in each box plot, from left to right, is n = 46; 87; 104; 152; 18; 31; 26; 12
857 (6mer+8mer), n = 51; 83; 117; 165 (6mer), n = 66; 79; 84; 82 (8mer), and n = 99; 60; 67;
858 84; 51 (no filaments). The respective median frequencies are 82% (6mer+8mer), 44%
859 (6mer), 81% (8mer), and 46% (no filaments). One-way ANOVA; ns=not significant; ***
860 P<0.001, **** P<0.0001. **(D)** Top, Western blots of cell lysates probed with anti-actin and

861 anti- α -tubulin antibodies under the same conditions as in (C). Molecular weight markers
862 are shown on the left. Bottom, respective quantification of actin protein levels (mean+SD).
863 Mean values (normalized to 1 for 6mer+8mer) are from 3-5 independent experiments.
864 One-way ANOVA; ns=not significant. **(E)** Atomic force microscopy nanoindentation on
865 cells under the conditions '6mer+8mer', '6mer' and '8mer' as described in (C). Left,
866 Example of an experimental force-indentation curve. Right and left arrows correspond to
867 the approach and retraction curves, respectively. The solid red lines represent the fits to
868 the viscoelastic model (see methods). The inset depicts the indentation of the cell, also
869 showing ventral and dorsal SFs in red. The image on the right shows the cantilever tip
870 indenting the dorsal membrane of a micropatterned wild-type cell. Right, box plots
871 showing the distributions of cell stiffness (E_0) and cell fluidity (β). E_0 values are plotted on
872 a log scale. The data points are plotted on top of the respective box plots; each data point
873 corresponds to one cell. On each box, the central mark indicates the median, and the
874 bottom and top edges of the box indicate the 25th and 75th percentiles, respectively. The
875 whiskers extend to the minimum and maximum values. The number of measurements in
876 each box plot, from left to right, is $n = 31, 29, 23$. The respective median cell stiffness
877 values are 656 Pa, 479 Pa, and 719 Pa, and the respective median cell fluidity values are
878 0.21, 0.23, and 0.19. One-way ANOVA for $\log(E_0)$ and for β ; ns=not significant; ** $P < 0.01$.
879

880 **Figure 9. Super-resolution structured illumination (SIM) microscopy of septin**
881 **filaments on SFs. (A-E)** Representative SIM micrographs of SEPT7 immunostained cells
882 co-stained for F-actin (phalloidin) (A-E), and additionally for α -actinin (C,D) or non-muscle
883 myosin heavy chain IIA (NMIIA) (E). Examples show septin filament localization to
884 perinuclear actin caps (A), arcs (B) and arc nodes (C), ventral nodes (D), and ventral SFs
885 (E, cells i-iv). The insets adjacent to the full field-of-views depict regions of interest (solid
886 outlined boxes) shown at high magnification. Dashed outlined boxes and their respective
887 insets show specific features at higher magnification. Scale bars in all large fields of views,
888 10 μm . Scale bars in insets, 1 or 2 μm as indicated. **(F-I)** Fiber width measurements and
889 real size estimations from SIM images. Box plots in (F) depict the distributions of
890 measured widths, as the full width at half maximum (FWHM), of microtubules (MT) (inset
891 shows an example SIM image of MTs) and septins associated with peripheral SFs
892 ("plasma membrane"), perinuclear actin caps ("cap"), arc and ventral actin nodes
893 ("aster"), arcs and ventral SFs; widths from thin and thick ventral septin fibers were plotted
894 separately. The data points are plotted on top of the respective box plots; data points
895 correspond to width measurements at multiple positions along MT and septin fibers and
896 in multiple MT and septin fibers per cell in a total of 10 cells for MT and 10 cells for septin
897 fiber measurements. On each box, the central mark indicates the median, and the bottom
898 and top edges of the box indicate the 25th and 75th percentiles, respectively. The
899 whiskers extend to the minimum and maximum values. The number of measurements in
900 each box plot, from left to right, is $n = 180, 123, 175, 88, 184, 330, 114$. The respective
901 median values are 115 nm, 123 nm, 137 nm, 131 nm, 133 nm, 134 nm, and 231 nm.

902 Kruskal-Wallis test; ns=not significant; **** P<0.0001. (G) Numerical simulations of the
903 expected FWHM in SIM images ("image diameter") as a function of the real fiber diameter.
904 The curve was generated from the convolution of a Gaussian point spread function (PSF)
905 of 115 nm with an increasing fiber size. Fiber sizes above ~200 nm scale linearly with the
906 image sizes. These simulations were used together with FWHM measurements in SIM
907 images (F) to estimate an upper width limit for septin fibers associated with the different
908 types of SFs (H). These estimations were then compared to the real width ranges one
909 expects from IgG antibody-decorated septins organizing as single or double filaments (I).
910 Primary and fluorophore (cyan asterisk)-conjugated secondary antibodies are depicted in
911 green and magenta, respectively. The primary SEPT7 antibody used in our
912 immunostainings binds the very C-terminus of SEPT7. The narrow and wide spacings of
913 paired filaments, the presence of homodimeric coiled coils for SEPT2, SEPT6 and
914 SEPT7, and of heterodimeric coiled coils for SEPT6 and SEPT7 are based on
915 experimental evidence from (de Almeida Marques et al., 2012; Leonardo et al., 2021; Low
916 and Macara, 2006; Sala et al., 2016). **(J)** Scatter dot plots of length distributions for septin
917 fibers on the indicated types of SFs. Bars depict median values. The number of
918 measurements in each plot, from left to right, is n = 151, 97, 227, 249. The respective
919 median values are 2.8 μm , 1.3 μm , 2.0 μm , and 3.8 μm .

920

921 **Figure 10. Septin filaments are closely apposed to the plasma membrane, are**
922 **largely immobilized on actin stress fibers, and can mediate actin-membrane**
923 **anchoring. (A)** Representative confocal micrographs of SEPT7 immunostained cells co-
924 stained for F-actin and non-muscle myosin heavy chain IIA (NMIIA). Cells were either
925 extracted after fixation (left panel) or were live-extracted right before fixation (right panel).
926 Scale bars, 10 μm . **(B)** Schematic of the metal-induced energy transfer (MIET) assay in
927 cells for probing fluorophore (mApple or AlexaFluor 568) distances from a gold-coated
928 coverslip using fluorescence lifetime measurements. **(C-E)** C depicts representative
929 examples of lifetime decay traces for SEPT9_i3-mApple on glass and in the presence of
930 gold (left) and for SEPT9_i3-mApple and SEPT9_i3-mApple-CAAX in the presence of
931 gold (right). The solid lines represent the numerical fits, showing the lifetime reduction
932 due to the MIET process. The calculated lifetime-distance dependence for SEPT9_i3-
933 mApple (D, see methods) was used to calculate the distance of SEPT9_i3-fused mApple,
934 with or without the CAAX lipid anchor, from the coverslip (E). Lifetime decay traces and
935 lifetime-distance dependence curves for GAP43-mApple (plasma membrane) and
936 AF568-phalloidin (F-actin) are shown in Fig. S5A,B. Box plots in (E) depict the
937 distributions of calculated distances for SEPT9_i3-mApple-CAAX, SEPT9_i3, GAP43-
938 mApple (plasma membrane, PM) and AF568-phalloidin (F-actin). The data points are
939 plotted on top of the respective box plots; each data point corresponds to one cell. On
940 each box, the central mark indicates the median, and the bottom and top edges of the
941 box indicate the 25th and 75th percentiles, respectively. The whiskers extend to the
942 minimum and maximum values. The number of measurements in each box plot, from left

943 to right, is $n = 13, 11, 8, 8$. The respective median values are 90 nm, 88 nm, 86 nm, and
944 115 nm. One-way ANOVA; ns=not significant; *** $P < 0.001$. **(F)** TIRF images of SEPT2-
945 msfGFP 8mer-9_i1 (top row) and F-actin (bottom row), either co-polymerized on top of a
946 supported lipid bilayer (SLB), or co-polymerized in solution to form preformed bundles
947 that were then flushed onto the supported lipid bilayer. The supported lipid bilayer was
948 composed either of 5% of PI(4,5)P₂, a septin-interacting lipid, and 95% DOPC (left
949 panels), or 100% DOPC (right panels). Due to the shallow penetration depth (~100 nm)
950 of TIRF together with the absence of crowding agents, only truly membrane-associated
951 structures are visible. Scale bar, 5 μm . **(G-K)** Septins are primarily immobilized and
952 confined on actin stress fibers but also undergo very slow lateral free diffusion in the
953 vicinity of the plasma membrane. (G-I) Left: Super-resolution PALM intensity images of
954 mEos2-Actin (G), SEPT9_i3-mEos3.2 (H) and SEPT9_i3-mEos3.2-CAAX (I) in mouse
955 embryonic fibroblasts obtained from a sptPALM sequence (50 Hz, >80 s). Insets: low
956 resolution images of GFP-actin (G) or EYFP-paxillin (H-I), which were co-expressed for
957 FA labelling. Scale bars, 3 μm . Right: color-coded trajectories overlaid on FAs labelled by
958 EYFP-paxillin or on FAs and SFs labelled by GFP-actin (grayscale) show the diffusion
959 modes: free diffusion (yellow), confined diffusion (green) and immobilization (red). (J)
960 Distributions of the diffusion coefficient D computed from the trajectories of mEos2-actin
961 (blue), SEPT9_i3-mEos3.2 (magenta) and SEPT9_i3-CAAX-mEos3.2 (light magenta)
962 obtained outside FAs, are shown in a logarithmic scale. The gray area including D values
963 inferior to $0.011 \mu\text{m}^2 \cdot \text{s}^{-1}$ corresponds to immobilized proteins. Values represent the
964 average of the distributions obtained from different cells. (K) Fraction of mEos2-actin
965 (blue), SEPT9_i3-mEos3.2 (magenta) and SEPT9_i3-mEos3.2-CAAX (light magenta)
966 undergoing free diffusion, confined diffusion or immobilization outside FAs. Values
967 represent the average of the fractions obtained from different cells (error bars: SEM).
968 Results for SEPT9_i3-mEos3.2 (14 cells) correspond to pooled data from two
969 independent experiments with n , the number of trajectories analyzed: SEPT9_i3-
970 mEos3.2 $n_{\text{SEPT9}_i3} = 72,720$. Results for mEos2-actin (9 cells) and SEPT9_i3-mEos3.2-
971 CAAX (5 cells) correspond each to data from one experiment with n , the number of
972 trajectories analyzed: mEos2-actin $n_{\text{actin}} = 34,715$; SEPT9_i3-mEos3.2-CAAX n_{SEPT9_i3-}
973 $\text{CAAX}} = 37,339$. Statistical significance in (K) was obtained using two-tailed, non-parametric
974 Mann–Whitney rank sum test. The different conditions were compared to the SEPT9_i3-
975 mEos3.2 condition. The resulting P values are indicated as follows: *** $P < 0.001$; ****
976 $P < 0.0001$. **(L)** Working model supported by the results of this study. Septins in cells
977 organize as paired, octamer-based filaments mediating actin-membrane anchoring.
978

979 **Materials and methods**

980

981 **Design of septin fusions for the tripartite split-GFP complementation assay.** For the
982 tripartite complementation assay to report SEPT-SEPT interactions with stringency, the
983 amino acid linker length between SEPT and the $\beta 10$ - and $\beta 11$ -strands should not be too

984 short in order to allow for the necessary proximity and flexibility for the β 10- and β 11-
985 strands to orient in an antiparallel fashion for complementing GFP1-9, but it should be
986 short enough to minimize reporting longer-range interactions. We used fluorescence
987 imaging to test the dependence of split-GFP complementation on the linker length and on
988 the position of the β 10- and β 11-tags by screening different homo- and hetero-septin
989 combinations as shown in Fig. S2A-C. All the combinations we tested resulted in
990 fluorescence, reflecting the inherent flexibility of the N- and C-termini of SEPT2, 7 and 9.
991 To allow for the most stringent complementation, we chose to use C-terminal fusions with
992 14-residue linkers for SEPT2- β 10- and - β 11 tags and for SEPT9- β 10- and - β 11 tags, and
993 N-terminal fusions with 14-residue linkers for β 10- and β 11-SEPT7 tags. This short linker
994 is comparable in length to the 10-residue-long β 10- and β 11-strands and thus long
995 enough to allow the antiparallel arrangement of the latter. Protein structure models of
996 human septin hexamers and octamers bearing full-length β 10- and β 11-tagged septins
997 (see method section "Modeling of human septin complexes") confirmed the efficiency of
998 GFP complementation for the final chosen linker length and β 10/ β 11-tag positioning (Fig.
999 2A,B; Fig. 3A,B; Fig. 5G,H).

1000

1001 **Plasmids and cloning.** Septin and msfGFP cDNAs were as described in (Iv et al., 2021).
1002 mApple and sfCherry2 cDNAs were PCR-amplified from Addgene plasmids #54862 and
1003 #83031, respectively. Three types of mammalian expression plasmids were used in this
1004 study. A pCMV backbone (Clontech) was used for the expression of full-length fluorescent
1005 protein (msfGFP/mApple) fusions. A pcDNA3.1 backbone (ThermoFisher Scientific), also
1006 with a CMV promoter, was used for the expression of β 10- or β 11-tagged septins. Finally,
1007 a pTRIP TRE Bi vector, modified from pTRE-Tight-BI (Takara-Bio) (Koraichi et al., 2018),
1008 bearing a bidirectional tetracycline response element (TRE) promoter and an IRES-
1009 TagBFP cassette downstream β 10-tagged septins, was used for the doxycycline-
1010 inducible co-expression of β 10- and β 11-tagged septins (Fig. S2D,E). pCMV and pTRIP
1011 TRE Bi plasmids were used for all results presented in the figures. The pcDNA3.1
1012 plasmids were used only for the initial screening (Fig. S2A-C).

1013 All pCMV plasmids, SEPT2 constructs in pTRIP TRE Bi plasmids and all interface
1014 mutants in pTRIP TRE Bi plasmids were cloned using seamless cloning (In-Fusion HD
1015 Cloning Plus Kit from Takara Bio, 638910). All pcDNAs and all wild-type SEPT7 and
1016 SEPT9-containing constructs in pTRIP TRE Bi plasmids were generated with classical
1017 cloning. In this latter case, DNA fragments were amplified by PCR using the PCR Master
1018 Mix from ThermoFisher Scientific (K0171), TaqFast DNA polymerase (Applied Biological
1019 Materials G277) or Phusion High-Fidelity DNA Polymerase (New England Biolabs
1020 M0530S) and ligated into double digested plasmids with the Rapid DNA Ligation Kit from
1021 ThermoFisher Scientific (K1422). pCMV constructs were cloned into a NheI/BamHI
1022 linearized vector. pcDNA constructs were cloned into a NheI/XbaI linearized vector.
1023 pTRIP TRE Bi constructs were cloned in two steps: first the β 10-tagged septins were
1024 cloned into a SacII/NheI digested vector, then the β 11-tagged septins were cloned into a

1025 NdeI/XbaI digested vector carrying the β 10-tagged septin. The starting methionine of
1026 septin sequences is included in the N-terminal β 10- and β 11-tagged versions.
1027 Bacterial expression plasmids for generating wild-type SEPT2-msfGFP hexamers and
1028 octamers-9_i3 were described in (Iv et al., 2021) and are available through Addgene
1029 (#174492, 174498, 174499, 174501). pNEA-vH plasmids for the bacterial expression of
1030 SEPT2NCmut-msfGFP and SEPT2-sfCherry2 were generated using seamless cloning
1031 following the same strategy described in (Iv et al., 2021). All primers for seamless cloning
1032 were Cloning Oligo (<60 bp) or EXTREmer (>60 bp) synthesis and purification quality
1033 from Eurofins Genomics, Germany and are listed in Table S1. All restriction enzymes
1034 were FastDigest enzymes from Thermo Scientific or from New England Biolabs. All
1035 plasmids were verified by sequencing (Eurofins Genomics, Germany) after each cloning
1036 step. We have deposited all plasmids with the nonprofit repository Addgene. Note that
1037 the SEPT9_i1NCmut in this study is the same as the SEPT9_i1NCmut2 in (Kuzmic et al.,
1038 2022). Plasmid mCherry-SEPT9_i1 was from Addgene (#71622).

1039
1040 **Cell lines, cell culture and transfection.** U2OS osteosarcoma cells for the expression
1041 of full-length fluorescent protein septin fusions were from ATCC (HBT-96). For the
1042 inducible co-expression of β 10- and β 11-tagged septins in the context of the tripartite
1043 split-GFP complementation system, we generated an inducible U2OS-Tet-On-GFP1-9
1044 cell line which expresses constitutively a GFP1-9 fragment and an anti-GFP VHH
1045 intrabody that enhances split-GFP fluorescence. To generate this cell line, U2OS cells
1046 were successively transduced with lentiviruses encoding rtTA, GFP1-9 (Addgene
1047 #130271) and anti-GFP VHH G4 (Addgene #182236) and tested for complementation
1048 efficiency using transient expression of a GFP10-zipper-GFP11 domain (Koraïchi et al.,
1049 2018). One additional round of transduction with GFP1-9 lentivirus led to an optimized
1050 U2OS-Tet-On-GFP1-9 cell line that showed 80% GFP positive cells upon expression of
1051 the GFP10-zipper-GFP11 domain. An IRES-TagBFP cassette downstream β 10-tagged
1052 septins was used for monitoring septin expression. Cells were maintained in McCoy's
1053 medium (Gibco 16600082) supplemented with 10% fetal bovine serum (Dominique
1054 Dutscher S181H), 100 U/mL penicillin and 100 μ g/mL streptomycin antibiotics (P4333,
1055 Sigma) in a humidified atmosphere at 37°C containing 5% CO₂.

1056 Transfections with pcDNAs, for the screening of β 10- and β 11-tag combinations (Fig.
1057 S2A-C), were performed 16 h prior to immunostainings using jetPRIME (PolyPlus
1058 101000015). To obtain single cells for imaging, 50x10³ U2OS-Tet-On-GFP1-9 cells were
1059 typically grown on 18 mm coverslips (Knittel Glass MS0010), previously cleaned by
1060 sonication in 70% ethanol, and placed into a 12-well plate a day prior to the day of
1061 transfection, for allowing an optimal number of cells to attach and spread. A total of 0.4
1062 μ g of DNA and a 4:1 ratio of jetPRIME (μ L) : DNA (μ g) were used per reaction. To
1063 minimize septin overexpression artifacts, the total amount of DNA was composed by 30
1064 ng of β 10-septin, 30 ng of β 11-septin and 340ng of empty vector.

1065 Transfections with either pCMV or pTRIP TRE Bi plasmids and siRNAs were performed
1066 through electroporation using the Neon Transfection System (Thermo Fisher Scientific
1067 MPK5000). For pCMVs, a single 100- μ L reaction using 1.8×10^6 U2OS cells, 300 pmol of
1068 each siRNA and 6 μ g of each DNA was electroporated within the dedicated tip (Thermo
1069 Fisher Scientific, MPK10096). Electroporation parameters consisted in 4 pulses of 10 ms
1070 width and a voltage of 1230 V. The electroporated cells were then inoculated in 5 mL of
1071 culture medium without antibiotics, and immediately divided for native-PAGE, SDS-
1072 PAGE/western blots and immunostaining as follows: 3 mL in a 6-cm dish containing 2 mL
1073 of medium without antibiotics, 2 mL in a 6-cm dish containing 3 mL of medium without
1074 antibiotics, and 100 μ L in the well of a 12-well plate containing a 18-mm coverslip in 900
1075 μ L of medium without antibiotics, respectively. A satisfactory septin knockdown efficiency
1076 was achieved within 48-96 h after electroporation. Typically, immunostaining and protein
1077 extraction were performed 72h post electroporation.

1078 For pTRIP TRE Bi plasmids, a single 100- μ L reaction using 1.8×10^6 U2OS-Tet-On-GFP1-
1079 9 cells, 300 pmol of each siRNA and 6 μ g of each DNA was electroporated within the
1080 dedicated tip using the same electroporation parameters described previously. The
1081 electroporated cells were then inoculated in 5.5 mL of culture medium without antibiotics
1082 and immediately divided for SDS-PAGE/western blots, immunostaining and live cell
1083 imaging as follows: 5 mL in a 6-cm dish, 400 μ L in the well of a 12-well plate containing
1084 a 18-mm coverslip in 600 μ L of medium without antibiotics, and 200 μ L in the well of a
1085 24-well glass bottom plate (Cellvis, P24-1.5H-N) containing 800 μ L of medium without
1086 antibiotics, respectively. After either 48 h, for samples intended for live cell imaging or
1087 immunostainings, or 72 h, for samples for biochemical analysis, protein expression was
1088 induced using 1 μ g/mL of doxycycline (Sigma, D9891) for 16 h.

1089
1090 **Septin mutant phenotype classification.** The diffuse cytosolic vs non-diffuse
1091 phenotype classification analysis for mutant characterization with pCMV plasmids (Fig.
1092 2H; Fig. 4E; Fig. 6C; Fig. S4A) was done from 3 independent experiments. Transfected
1093 cells were fixed and co-stained for actin and α -tubulin. Each round of experiments was
1094 composed of the 30 first fluorescent cells found randomly in the sample, with the
1095 exception of one round containing 11 cells for msfGFP-SEPT7 and 8 cells for msfGFP-
1096 SEPT7Gmut1. Acquired images were classified as "diffuse cytosolic" in the presence of
1097 purely diffuse cytosolic signal or as "non-diffuse" in the presence of structure-like signal;
1098 no differentiation was applied for SF-, microtubule-, membrane-like or punctate signals in
1099 the latter case. The violin graphs representing the phenotype distributions show the mean
1100 intensity distribution calculated on the whole field of view from maximum intensity
1101 projections of all z-planes. The phenotype classification for reconstituted split-GFP
1102 fluorescence distribution (Fig. 2F; Fig. 4G,H; Fig. 6D; Fig. S4B) was identical, in terms of
1103 the used criteria and graph display, but the data was generated from 2 independent
1104 experiments, with each experimental round composed of the 20 first fluorescent cells, in
1105 live cell imaging, with the exception of one round containing 13 cells for SEPT7Gmut1-

1106 SEPT7Gmut1 and 9 cells for SEPT7Gmut2-SEPT7Gmut2. For the septin-actin
1107 colocalization analysis, the diffuse cytosolic vs non-diffuse phenotype sorting is displayed
1108 both as scatter dot plots and as a pie graph to highlight the diffuse cytosolic vs non-diffuse
1109 proportion from each condition. Bars in scatter dot plots depict means and error bars SD.
1110 Violin plots, scatter dot plots and pie graphs were prepared using GraphPad Prism. The
1111 number of cells used to assess the phenotypes for each condition is indicated in the
1112 respective legends.

1113

1114 **RNA interference.** Control synthetic small interfering RNA (siRNA) targeting the coding
1115 region of LacZ (5'-GCGGCUGCCGGAAUUUACC-3') and siRNA targeting the 3'UTR
1116 region of all SEPT9 mRNA variants (5'-GGAUCUGAUUGAGGAUAAA-3') were
1117 previously validated (Verdier-Pinard et al., 2017). The siRNA sequences targeting the
1118 3'UTR regions of SEPT2 and SEPT7 were 5'-ACACUUUCCUGGAUAAAA-3' and 5'-
1119 GCAUUUAGCUGUAUUCAUA-3', respectively. All siRNAs were designed to hybridize
1120 with 19-bp sequences in the 3'UTR regions of septin genes, thus knocking down
1121 endogenous septins while allowing the expression of the transfected plasmids. 21mer
1122 siRNAs, 20 nmol each, were synthesized with dTdT overhangs by Eurofins, and delivered
1123 as annealed and ready-to-use siRNA duplexes. siRNAs targeting the coding regions of
1124 non-muscle myosin heavy chain IIA (NMIIA) (5'-
1125 GCCACGCCAGAGAACGAGAAUGC-3') and non-muscle myosin heavy chain IIB
1126 (NMIIB) (5'-UCAAUAAAGCUCUGGAUAGGACCAA-3') were previously validated (Kage
1127 et al., 2022). 20 nmol of these 25mer siRNAs without overhangs were synthesized by
1128 Eurofins, and delivered as annealed and ready-to-use siRNA duplexes.

1129

1130 **SDS-PAGE and western blotting of cell lysates.** The dish containing the cells was
1131 placed on ice and the cells were washed twice with PBS, without Ca²⁺ and Mg²⁺, before
1132 being detached with 40 µL of ice-cold lysis buffer (10 mM Tris-HCl pH 7.5, 148.5 mM
1133 NaCl, 0.5 mM EDTA, 0.5% NP-40, 1x PhosSTOP Roche, 5x cOmplete protease inhibitor
1134 cocktail Roche, 1 mM DTT) using a cell scraper (TPP 99003). The lysate was collected
1135 in a 1.5 mL tube and incubated on ice for 30 min. The lysates were then centrifuged at
1136 20,000 g for 20 minutes at 4°C for removing cell debris. An aliquot of 6 µL was collected
1137 for protein quantification using the BCA Protein Assay (ThermoFisher Scientific 23227)
1138 and the remaining clarified lysates were kept at -20°C until SDS-PAGE analysis.

1139 The lysates were analyzed by 4-20% SDS-PAGE using Mini-PROTEAN TGX™ Precast
1140 Protein Gels (BioRad 4561095). Molecular mass markers were Precision Plus Protein All
1141 Blue Standards (BioRad 1610373) or Amersham ECL Rainbow Marker (Cytiva
1142 RPN800E). For the western blot, the gel, the PVDF Immobilon-P^{SQ} membrane (MERCK
1143 ISEQ85R), filter pads and filter papers were all incubated in transfer buffer (25 mM Tris,
1144 192 mM glycine and 20% of methanol) for 15 min before assembly in the Mini Trans-Blot
1145 transfer cell (BioRad 1703935). The transfer was done at 4°C for 16 h at 110 mA constant
1146 current. The transfer efficiency was checked by Ponceau S staining (Sigma P7170). The

1147 membrane was then blocked in a 3% w/v dry milk TBS-T solution (20 mM Tris-HCl pH7.5,
1148 200 mM NaCl and 0.1% v/v Tween20) for 90 min under constant agitation. Primary and
1149 secondary antibodies were diluted in the same blocking solution and incubated over the
1150 membrane for 90 and 60 min, respectively. In between antibody incubations, membranes
1151 were washed three times for 10 min with TBS-T, and the very last wash right before ECL
1152 detection was done only with TBS.

1153 The loaded amount of extracted protein in the gels was adapted depending on the
1154 expression promoter and the analyzed septin. For pCMV plasmids used to assess the
1155 knockdown efficiency (Fig. S2F-H), a total of 4 µg of extracted protein was used for
1156 detecting endogenous SEPT2, 8 µg for endogenous SEPT7, and 4 µg for endogenous
1157 SEPT9. For pTRIP TRE Bi plasmids, a total of 8 µg of extracted protein was used for all
1158 analysis. To detect specific septins, we used rabbit anti-SEPT2 (1:2500, Sigma
1159 HPA018481), rabbit anti-SEPT7 (1:200, Santa Cruz Biotechnology sc-20620) and rabbit
1160 anti-SEPT9 (1:4000, Proteintech 10769-1-AP). For detecting β10- and β11-tag
1161 expression, we used rabbit anti-β10 (1:5,000) and rabbit anti-β11 (1:5,000) (Koraichi et
1162 al., 2018). For detecting tubulin as a loading control, we used mouse anti-α-tubulin
1163 (1:2,500, Sigma T9026). For total actin level quantification (Fig. 8D), we used mouse anti-
1164 actin (1:1000, AC-40, Abcam ab11003). Secondary HRP-conjugated antibodies were
1165 either anti-rabbit-IgG (1:10,000, Cytiva GENA934) or anti-mouse-IgG (1:10,000, Cytiva
1166 GENA931). Chemiluminescent detection was performed with an Amersham ImageQuant
1167 800 imager (Cytiva 29399481) using Amersham ECL Select Western Blotting Detection
1168 Reagent (Cytiva RPN2235) diluted five times in Milli-Q water. The membrane was
1169 incubated with the diluted reagent for 30 s, and washed for 10 s in TBS right before image
1170 acquisition. Images were collected in time series mode every 10 s, for a total of 50 images,
1171 and processed with ImageQuantTL software for quantification of the band intensities to
1172 measure expression levels. Expression quantification graphs for assessing septin
1173 knockdown efficiency were prepared using GraphPad Prism and are shown as mean
1174 values (normalized to 1 for siCtrl) with the error bar representing the standard deviation.
1175 Data are from at least 3 independent siRNA treatments. Data distribution for total actin
1176 level quantification (Fig. 8D) was assumed to be normal but this was not formally tested.
1177

1178 **Native PAGE and western blotting of cell lysates.** The dish containing the cells was
1179 placed on ice and the cells were washed twice with PBS, without Ca²⁺ and Mg²⁺, before
1180 being detached with 40 µL of ice-cold native lysis buffer (80 mM PIPES pH 6.9, 2 mM
1181 MgCl₂, 4 mM EGTA, 0.2% saponin, 5x cOmplete protease inhibitor cocktail Roche). The
1182 lysate was collected in 1.5 mL tube and incubated on ice for 10 min. The lysates were
1183 then centrifuged at 14,000 g for 10 min at 4°C for removing cell debris. To prevent septin
1184 polymerization, clarified lysates were supplemented with NaCl, adding 10 µL of NaCl 5 M
1185 for each 100 µL of lysate. After 15 min of incubation on ice, the lysates were clarified in a
1186 second centrifugation step of 10 min, 14,000 g at 4°C. An aliquot of 12 µL was collected

1187 for protein quantification using the BCA Protein Assay (ThermoFisherScientific 23227),
1188 and the remaining clarified lysates were kept at -20°C until Native PAGE analysis.
1189 The lysates were analyzed by 4-16% Native PAGE using precast Bis-Tris Mini Protein
1190 Protein Gels (Invitrogen BN1003BOX) following the manufacturer's instructions. The
1191 molecular mass marker was NativeMark™ Unstained Protein Standard (Invitrogen,
1192 LC0725). For the western blot, the gel, the PVDF Immobilon-P^{SQ} membrane, filter pads
1193 and filter papers were all incubated in NuPAGE transfer buffer for 15 min before
1194 assembly. The transfer was done at 4°C for 16 h at 20 V constant voltage. The transfer
1195 efficiency was checked by destaining the membrane with an aqueous solution containing
1196 25% of methanol and 10% of acetic acid. The protein marker was identified and the
1197 membrane completely destained with pure methanol for 3 min. The membrane was then
1198 blocked and stained with the respective antibodies as described for SDS-PAGE western
1199 blots.

1200 The loaded amount of extracted protein in the gels was again adapted depending on the
1201 analyzed septin. A total of 10 µg of extracted protein was used for detecting endogenous
1202 or exogenously expressed SEPT2 and SEPT7, and 10 or 4 µg for endogenous or
1203 exogenously expressed SEPT9, respectively. To detect specific septins, we used mouse
1204 anti-SEPT2 (1:7,500, Proteintech 60075-1), rabbit anti-SEPT7 (1:200, Santa Cruz
1205 Biotechnology sc-20620) and rabbit anti-SEPT9 (1:2,000, Proteintech 10769-1-AP).
1206 Secondary HRP-conjugated antibodies were either anti-rabbit IgG (1:10,000, Cytiva
1207 GENA934) or anti-mouse IgG (1:10,000, Cytiva GENA931). Chemiluminescent detection
1208 was done with an Amersham ImageQuant 800 imager (Cytiva 29399481) using
1209 Amersham ECL Select Western Blotting Detection Reagent (Cytiva RPN2235) as
1210 described previously for SDS-PAGE western blot.

1211
1212 **Immunofluorescence.** Cells were fixed for 15 min with 4% paraformaldehyde (Electron
1213 Microscopy Sciences 15714) in 37°C-prewarmed cytoskeleton buffer (10 mM MES pH
1214 6.1, 150 mM NaCl, 5 mM EGTA, 5 mM MgCl₂, 5 mM glucose), followed by 2 x 5 min wash
1215 steps in phosphate-buffered saline (PBS) solution, and a subsequent permeabilization
1216 and blocking step with PBS containing 0.1% saponin and 1% IgG-free/protease free
1217 bovine serum albumin (BSA) (Jackson ImmunoResearch 001-000-161) for 1 h at RT.
1218 Cells were incubated successively with primary antibodies for 16 h at 4°C in a humidified
1219 chamber, followed by secondary Alexa Fluor-conjugated IgG antibodies combined with
1220 0.165 µM Alexa Fluor 647-phalloidin (ThermoFisher Scientific A22287) for 2 h at RT.
1221 Antibody solutions were prepared in PBS containing 0.1% saponin and 1% BSA, and 3 x
1222 10 min wash steps in the same buffer were performed in between antibody incubations.
1223 Coverslips with stained cells were washed 2 x 5 min in PBS and then mounted with 15
1224 µL Fluoromount (Sigma F4680) for image acquisition. Primary antibodies were rabbit anti-
1225 SEPT2 (1:500, Sigma HPA018481), rabbit anti-SEPT7 (1:500, IBL 18991), rabbit anti-
1226 SEPT9 (1:200, Proteintech 10769-1-AP), mouse anti-α-tubulin (1:10,000, Sigma T9026),
1227 mouse anti-paxillin (1:500, Merck Millipore 05-417). Secondary antibodies were donkey

1228 AlexaFluor488-conjugated anti-rabbit IgGs (1:500, Thermo Fisher Scientific A10037) and
1229 donkey AlexaFluor568-conjugated anti-mouse IgGs (1:500, Thermo Fisher Scientific
1230 A21206). For F-actin, SEPT9, and SEPT7 co-stainings, performed for the quantification
1231 of the number of ventral SFs in cells (see methods section "Quantification of the frequency
1232 of ventral SF presence upon septin perturbation"), rabbit anti-SEPT9 (1:200, Proteintech
1233 10769-1-AP) was combined with rat anti-SEPT7 (1:150, clone 10A7, described in (Kuzmic
1234 et al., 2022)). Secondary antibodies in this case were donkey AlexaFluor488-conjugated
1235 anti-rabbit IgGs (1:500, Thermo Fisher Scientific A10037) and goat AlexaFluor647-
1236 conjugated anti-rat IgGs (1:500, Thermo Fisher Scientific A21247), and were incubated
1237 together with 0.165 μ M Atto590-phalloidin (ATTO-TEC AD 590-81).

1238

1239 **Immunostaining after live-cell extraction vs after extraction post-fixation.** To live-
1240 extract cells (Fig. 10A), we incubated cells in 37°C-prewarmed cytoskeleton buffer
1241 containing 0.1% v/v Triton X-100 for 1 min, then replaced immediately with 37°C-
1242 prewarmed cytoskeleton buffer containing 4% paraformaldehyde (Alfa Aesar 43368) and
1243 fixed cells for 15 min. Cells were rinsed with PBS and incubated in a
1244 permeabilization/blocking solution of PBS containing 0.1% saponin and 5% goat serum
1245 (ThermoFisher Scientific 16210064) overnight at 4°C. Cells were incubated successively
1246 with primary antibodies for 2 h at RT in a humidified chamber, followed by secondary
1247 Alexa Fluor-conjugated IgG antibodies combined with 0.165 μ M Alexa Fluor 546-
1248 phalloidin (ThermoFisher Scientific A22283) for 1 h at RT. Antibody solutions were
1249 prepared in PBS containing 0.1% saponin and 5% goat serum, and 3 x 10 min wash steps
1250 in the same buffer were performed in between antibody incubations. Coverslips with
1251 stained cells were washed 2 x 5 min in PBS and then mounted with Fluoromount-G
1252 (Southern Biotech 0100-01) for image acquisition. Primary antibodies were rabbit anti-
1253 SEPT7 (1:400, IBL 18991) and mouse anti-non-muscle myosin heavy chain IIA (NMIIA)
1254 (1:200, abcam ab55456). Secondary antibodies were goat AlexaFluor488-conjugated
1255 anti-mouse IgGs (1:400, Thermo Fisher Scientific A11001) and goat AlexaFluor633-
1256 conjugated anti-rabbit IgGs (1:400, Thermo Fisher Scientific A21070). The respective
1257 control experiment, i.e. extracting cells post-fixation (Fig. 10A), involved fixing cells in
1258 37°C-prewarmed cytoskeleton buffer containing 4% paraformaldehyde for 15 min, then
1259 extracting cells with PBS containing 0.5% Triton X-100 and 5% goat serum for 10 min
1260 before overnight permeabilization/blocking and antibody incubations as described above.
1261 This last protocol was also used for the immunostainings shown in Fig. 9 and Fig. S1C,D
1262 using the additional primary antibody mouse anti- α -actinin-1 (1:200, Thermo Scientific
1263 clone BM 75.2). Cells shown in Fig. 9, Fig. 10A and Fig. S1C,D were plated on fibronectin-
1264 coated coverslips and left to attach and spread for 6 h before immunostainings. Human
1265 plasma fibronectin was from Millipore (FC010) and was used at 20 μ g/mL in 100 mM
1266 bicarbonate buffer pH 8.5 for coating coverslips overnight at 4°C.

1267

1268 **Split-GFP complementation in cells using recombinant purified GFP1-9.**
1269 Recombinant GFP1-9 expression was achieved from a pET-28a (+) vector coding for
1270 GFP1-9 OPT (Cabantous et al., 2013) with an in-frame 6xHis coding sequence (Addgene
1271 #182240). For protein expression, the plasmid was transformed into *Escherichia coli*
1272 BL21(DE3) (New England Biolabs C2527). A 3 mL overnight culture was inoculated into
1273 50 mL of Luria-Bertani (LB) medium containing 35 µg/ml kanamycin and was grown at
1274 37°C to $A_{600nm} \sim 0.6$. The temperature was reduced to 25°C prior to induction with 0.1 mM
1275 isopropyl β-D-thiogalactoside (IPTG) for 24 h. Bacterial cultures were collected by
1276 centrifugation at 4,200 g for 10 min. Cell pellets were resuspended in 20 mL lysis buffer
1277 (150 mM NaCl, 150 mM Tris-HCl pH 7.2, 5% v/v glycerol, 0.2 mM TCEP, 10 mM
1278 imidazole) and lysed by sonication on ice with a 0.5-inch diameter probe with 30 sec ON,
1279 30 sec OFF pulses for 4 min total. The lysate was centrifuged at 30,000 g for 20 min. The
1280 supernatant was loaded on a column with 1 mL of TALON® Metal Affinity Resin (Takara
1281 Bio 635503) that was equilibrated with the lysis buffer. After two washes with ten volumes
1282 of lysis buffer, recombinant GFP1-9 was eluted with the elution buffer (200 mM imidazole
1283 in the same buffer). Recombinant GFP1-9 fractions were pooled and dialyzed in TNG
1284 buffer (150 mM NaCl, 50 mM Tris-HCl pH 7.2, 5% v/v glycerol). Protein concentration was
1285 determined with the Bio-Rad Protein Assay (Bio-Rad 5000002).
1286 For split-GFP complementation in cells, wild type U2OS cells were treated with SEPT2
1287 siRNA and also co-transfected with SEPT2-β10- and SEPT2-β11-encoding pCMV
1288 plasmids, and plated on 18-mm coverslips as described above for immunostainings. 48
1289 h post-electroporation, cells were fixed and permeabilized as described for
1290 immunofluorescence, and incubated with the recombinant purified GFP1-9 solution (0.2
1291 mg/mL in TNG buffer) diluted 2-fold in the permeabilization buffer for 4h at RT followed
1292 by overnight incubation at 4°C. After 2 x 10 min wash steps, cells were stained with 0.165
1293 µM Alexa Fluor 647-phalloidin for 1h at RT. Fluorescence images of SEPT2-SEPT2
1294 reconstituted GFP in the phalloidin-stained cells were acquired as described in the
1295 confocal fluorescence microscopy methods section.
1296
1297 **Myosin-II-dependence of septin recruitment to stress fibers.** For septin costainings
1298 with non-muscle myosin heavy chain IIA (NMIIA) and non-muscle myosin heavy chain IIB
1299 (NMIIB), wild-type U2OS cells were treated with siLacZ siRNA and prepared for
1300 immunofluorescence as described in the respective sections. Primary antibodies were
1301 rabbit anti-NMIIA (1:400, BioLegend 909801), rabbit anti-NMIIB (1:200, Cell Signaling
1302 3404), and rat anti-SEPT7 (1:150, clone 10A7, described in (Kuzmic et al., 2022)), and
1303 were incubated for 4 h at RT. Secondary antibodies were donkey AlexaFluor488-
1304 conjugated anti-rabbit IgGs (1:400, Thermo Fisher Scientific A10037) and goat
1305 AlexaFluor568-conjugated anti-rat IgGs (1:400, Thermo Fisher Scientific A11077), and
1306 were incubated together with 0.165 µM Alexa Fluor 647-phalloidin (ThermoFisher
1307 Scientific A22287) for 1 h at RT.

1308 To examine septins in the absence of a given NMII isoform, CRISPR-mediated NMIIA
1309 and NMIIB knock-out (KO) U2OS lines (Kage et al., 2022), a kind gift from Frieda Kage
1310 and Henry Higgs (Dartmouth College), were treated with siLacZ siRNA and
1311 immunostained for SEPT7, F-actin and the myosin isoform that is not knocked-out, i.e.,
1312 for NMIIB in the NMIIA KO line and for NMIIA in the NMIIB KO line, using the same
1313 antibodies described in this section. NMIIA KO and NMIIB KO U2OS lines were cultured
1314 as wild-type U2OS cells.

1315 Given that NMIIA is by far the most abundant NMII in U2OS cells (Kage et al., 2022) and
1316 that the efficiency of knocking down NMIIB (~ 96-98%) was much higher than knocking
1317 down NMIIA in our hands (~ 60-76%), we chose to treat NMIIA KO cells with NMIIB siRNA
1318 to examine septins under conditions of minimal presence of NMII in U2OS cells.
1319 Treatment with NMIIB siRNA was performed as described in (Kage et al., 2022) but with
1320 electroporation. SDS-PAGE and western blotting in the NMIIA KO and NMIIB KO cell
1321 lysates was performed as described in the respective methods section using the same
1322 rabbit NMII antibodies described in this section at 1:1000 each, rabbit anti-SEPT7 (1:200,
1323 Santa Cruz Biotechnology sc-20620) and mouse anti- α -tubulin (1:2,500, Sigma T9026).

1324

1325 **Quantification of the frequency of ventral SF presence upon septin perturbation.**

1326 The frequency of ventral SF presence in wild type U2OS cells treated with siLacZ siRNA
1327 (6mer+8mer), with SEPT9 siRNA (6mer), with SEPT7 siRNA and also transfected with
1328 msfGFP-SEPT7Gmut2 (8mer), and in U2OS cells treated with SEPT2 siRNA and also
1329 transfected with SEPT2NCmut-msfGFP (no filaments), was quantified from at least 4
1330 independent experiments (Fig. 8C). Electroporated cells were either incubated with SiR-
1331 actin for live cell imaging (2 independent experiments for 6mer+8mer, 6mer and 8mer
1332 conditions) or fixed and co-stained for F-actin, SEPT9 and SEPT7 (from 2 to 6
1333 independent experiments for 6mer+8mer, 6mer, 8mer and 'no filaments' conditions).
1334 Each round of experiments was composed of at least 20 and 10 fields of view for live and
1335 fixed cell imaging, respectively. Only cells with most of their surface in the acquired field
1336 of view were considered for the quantification, and were classified regarding the
1337 presence, in the case of at least one detected ventral SF, or absence of ventral SFs based
1338 on the phalloidin or SiR-actin signal; no differentiation was applied for the density/number
1339 of ventral SFs, or the presence of other SF subtypes, as no clear effect could be detected
1340 in the latter. Each dot in the scatter plots represents the percentage of cells presenting
1341 ventral SFs in a given round of experiments. Bars in scatter dot plots depict means and
1342 error bars SD. Scatter dot plots were prepared using GraphPad Prism. The number of
1343 cells used to assess the phenotypes for each condition is indicated in the respective
1344 legends. Data distribution was assumed to be normal but this was not formally tested. For
1345 the quantification of the percentage of SEPT9 siRNA-treated cells containing SEPT7 vs
1346 SEPT9-decorated SFs, two rounds of experiments using fixed cells were used. Only 2-
1347 3% of SEPT9 siRNA-treated cells showed SEPT7-decorated SFs, and these same SFs

1348 contained also SEPT9, corresponding to the small percentage of cells where SEPT9 was
1349 not knocked down.

1350

1351 **Confocal fluorescence microscopy of cells and image processing.** For live cell
1352 imaging, right before microscopy and due to the absence of CO₂ control on our
1353 microscope setup, the culture medium was exchanged by Leibovitz medium (Gibco
1354 21083027) supplemented with 10% fetal bovine serum and antibiotics. Cells were kept at
1355 37°C in a heating chamber (Okolab H301-T-UNIT-BL). Fluorescence images of live or
1356 fixed cells were acquired using a spinning disk unit (CSU-X1-M1 from Yokogawa)
1357 connected to the side-port of an inverted microscope (Eclipse Ti2-E from Nikon
1358 Instruments) using a Nikon Plan Apo ×100/1.45 NA oil immersion objective lens, 488-
1359 561- and 641-nm laser lines (Coherent) and an iXon Ultra 888 EMCCD camera
1360 (1024×1024 pixels, 13×13 μm pixel size, Andor, Oxford Instruments) resulting in an image
1361 pixel size of 65 nm. Z-stacks were acquired with a Δz interval of 0.4 μm. Exposure times
1362 were in the range of 0.5-3.0 s depending on the exact condition. For the non-diffuse vs
1363 diffuse cytosolic phenotype classification for septin mutant characterization, acquisition
1364 parameters were kept the same among imaging sessions. For actin or microtubule co-
1365 labeling in live cells, cells were incubated for 30 min with 0.5 μM of SiR-actin or 60 min
1366 with 0.5 μM of SiR-tubulin and 10 μM of verapamil in culture medium (SiR Cytoskeleton
1367 Kit, Spirochrome SC006).

1368 Images were processed with the open-source image processing software ImageJ/Fiji. All
1369 shown images, except for the ones used for the septin-actin co-localization analysis that
1370 were acquired as single z-planes, are maximum intensity projections of two consecutive
1371 z-planes contrasted manually in order to optimize the image display. For septin-actin co-
1372 localization measurements, acquired channels of single z-planes, for septin and actin,
1373 were individually processed as follows: images were subjected to automatic contrast
1374 enhancement, allowing 0.1% of saturated pixels, then to a blurring with a Gaussian filter
1375 of radius 1.0 and a subsequent background subtraction using a rolling ball radius of 7
1376 pixels. A manual intensity threshold was used when calculating Pearson and Manders
1377 co-localization coefficients, using the JACoP plugin for ImageJ (Bolte and Cordelières
1378 2006).

1379 Images shown in Fig. 10A and Fig. S1C,D were acquired on a Zeiss LSM 710 laser
1380 scanning confocal microscope using a PlanApochromat 100x/1.4 NA oil immersion
1381 objective lens, 488- 543- and 633-nm laser lines for excitation, with all channels at 1AU
1382 for the pinholes. Z-stacks were acquired with a Δz interval of 0.48 μm. All shown images
1383 are single z-planes and were processed with ImageJ/Fiji.

1384

1385 **Super-resolution structured illumination microscopy.**

1386 **Sample preparation and image acquisition.** Cells for super-resolution structured
1387 illumination (SIM) microscopy were plated on high precision (170 ± 5 μm thick) 18x18mm
1388 glass coverslips from Zeiss (474030-9000-000) and prepared for immunostainings as

1389 detailed in the section "Immunostaining after live-cell extraction vs after extraction post-
1390 fixation"; all images shown in Fig. 9 and Fig. S4K employ extraction post-fixation. For
1391 microtubule stainings used for microtubule width measurements in Fig. 9F,H, cells were
1392 fixed with -20°C-prechilled methanol for 2 min at -20°C and rinsed with PBS before
1393 overnight permeabilization/blocking and antibody incubations as described in the above
1394 section. Primary antibodies were mouse tubulin (1:1,000, Sigma T9026) and rabbit anti-
1395 SEPT7 (1:500, IBL 18991). Secondary antibodies were goat AlexaFluor488-conjugated
1396 anti-mouse IgGs (1:400, Thermo Fisher Scientific A11001) and goat AlexaFluor633-
1397 conjugated anti-rabbit IgGs (1:400, Thermo Fisher Scientific A21070). Images in Fig. 9
1398 were acquired on a Zeiss Elyra PS.1 super-resolution microscope using an alpha
1399 PlanApochromat 100x/1.46 NA DIC M27 Elyra oil immersion objective lens, 488-, 561-,
1400 and 642-nm laser lines for excitation and respective BP495-550, BP570-620 and LP655
1401 emission filters. Z-stacks were acquired with a Δz interval of 0.101 μm . Images were
1402 processed and channel-aligned with the Zeiss ZEN Black software. Images in Fig. S4K
1403 were acquired on a DeltaVision OMX SR (Leica Microsystems/Cytiva) super-resolution
1404 microscope using an Olympus PlanApo N 60x/1.42 NA oil immersion objective lens, 488-
1405 and 640-nm laser lines for excitation and respective 528/48 and 683/40 emission filters.
1406 Z-stacks were acquired with a Δz interval of 0.125 μm . Images were processed and
1407 channel-aligned with the DeltaVision softWoRx 7.0.0 software. All shown images are
1408 single z-planes and were prepared with ImageJ/Fiji.

1409 **Septin fiber diameter and length measurements.** Full width at half maximum (FWHM)
1410 measurements for measuring the diameter of microtubules (MT) and septin fibers in SIM
1411 images (Fig. 9F,H) were made with a custom-generated Matlab code
1412 (FilamentAnalysis.mlx), the source code of which is available at
1413 [gitHub.com/cchandre/Polarimetry](https://github.com/cchandre/Polarimetry). A line was drawn perpendicular to the axis of the MT
1414 or to the long axis of the septin fiber, and the FWHM was extracted from the intensity
1415 profile using the *findpeaks* Matlab function. We measured the width of MTs and septin
1416 fibers at multiple positions along their length and in multiple microtubules and multiple
1417 septin fibers for each SF type per cell. Box plots depicting the distribution of FWHM
1418 measurements (Fig. 9F) were prepared using GraphPad Prism (one data point
1419 corresponds to one width measurement). The central mark indicates the median, and the
1420 bottom and top edges of the box indicate the 25th and 75th percentiles, respectively. The
1421 whiskers extend to the minimum and maximum values. The number of measurements
1422 per condition (MT or SF type) is indicated in the respective legend. A Kruskal-Wallis test
1423 followed by a multiple comparison test was used for comparing the distributions.

1424 Length measurements were implemented in the same custom-generated code. A line
1425 was drawn parallel to the long axis of the septin fiber and the length extracted with the
1426 *curveLength* Matlab function. We measured the length of multiple septin fibers for each
1427 SF type per cell. Scatter dot plots depicting the distribution of length measurements (Fig.
1428 9J) were prepared using GraphPad Prism (one data point corresponds to one length

1429 measurement). The number of measurements per SF type is indicated in the respective
1430 legend. Bars depict median values.

1431
1432 **Numerical simulations for fiber size estimation.** Numerical simulations of the expected
1433 FWHM in SIM images ("image diameter" in Fig. 9G) as a function of the real fiber diameter
1434 ("fiber diameter" in Fig. 9G) were made with a custom-generated Matlab code
1435 (Convolution_1D.m), the source code of which is available at
1436 [gitHub.com/cchandre/Polarimetry](https://github.com/cchandre/Polarimetry). A Gaussian point spread function (PSF) was used,
1437 and the curve was generated from the convolution of this PSF with an increasing fiber
1438 diameter size, using the conv Matlab function. Assuming a real antibody-decorated MT
1439 diameter size of ~60 nm (Weber et al., 1978), the convolution curve permits to deduce
1440 the PSF size from the measured FWHM in isolated microtubule fibers (the median value
1441 is used). This PSF size being linearly dependent on the emission wavelength, it is then
1442 rescaled to account for the wavelength difference used in MT vs septin imaging: MTs
1443 and septins were imaged at 488 and 642 nm, respectively, for SIM in Fig. 9, whereas MTs
1444 and septins for SIM in Fig. S4K were imaged at 640 and 488 nm, respectively. To predict
1445 the real width of the respective septin fiber diameters (Fig. 9H), the convolution curve was
1446 finally used for the estimated PSF, using as input the measured septin FWHM.

1447
1448 **Production and purification of recombinant human septin complexes.** Wild-type
1449 nonfluorescent and SEPT2-msfGFP hexamers and octamers-9_i3, SEPT2NCmut-
1450 msfGFP hexamers and octamers-9_i3, and SEPT2-sfCherry2 octamers-9_i3 were
1451 produced and purified as follows. Plasmids expressing SEPT2, SEPT2-msfGFP or
1452 SEPT2NCmut-msfGFP, and plasmids co-expressing SEPT2, SEPT2-msfGFP or
1453 SEPT2NCmut-msfGFP and SEPT6, were co-transformed with plasmids co-expressing
1454 SEPT6 and SEPT7 (Addgene #174499), or SEPT7 and SEPT9_i3 (Addgene #174501),
1455 for generating recombinant nonfluorescent, SEPT2-msfGFP, or SEPT2NCmut-msfGFP
1456 hexamers and octamers-9_i3 (lv et al., 2021). Plasmids co-expressing SEPT2-sfCherry2
1457 and SEPT6 were co-transformed with plasmids co-expressing SEPT7 and SEPT9_i3
1458 (Addgene #174501) to generate recombinant SEPT2-sfCherry2 octamers-9_i3. The N-
1459 terminus of SEPT2 is tagged with a His₆-tag, and the C-terminus of SEPT7 (for isolation
1460 of hexamers), or the C-terminus of SEPT9 (for isolation of octamers), is tagged with a
1461 Strep-tag. A purification scheme comprising a Strep-Tactin affinity column to capture
1462 Strep-tagged complexes, followed by a nickel affinity column to retain the Strep-tagged
1463 complexes that also bear His₆-tagged septins isolates hexamers and octamers (lv et al.,
1464 2021).

1465 Co-transformed *E. coli* BL21(DE3) were selected on LB agar plates with carbenicillin and
1466 spectinomycin each at 100µg/mL. A single colony was selected to prepare an overnight
1467 LB medium preculture at 37°C with antibiotics at 100 µg/mL. Terrific broth with antibiotics
1468 at 50 µg/mL, typically 3.5-5 L, was inoculated with the pre-culture and incubated at 37°C.
1469 Bacteria were left to grow to $A_{600nm} \sim 0.6-0.8$ before inducing expression with 0.5 mM

1470 IPTG for overnight expression at 17°C. The culture was stopped by centrifuging at 3,400
1471 g for 15 min and 4°C, and the supernatants were pooled and further centrifuged at 5,000
1472 g for 10 min and 4°C. Bacteria pellets were stored at -20°C until protein purification.
1473 Bacteria expressing msfGFP- and sfCherry2-tagged septins yield yellow-greenish and
1474 pink-reddish pellets, respectively.

1475 On the day of purification, the pellet was resuspended in ice-cold lysis buffer (50 mM Tris-
1476 HCl pH 8, 300 mM KCl, 5 mM MgCl₂, 0.25 mg/mL lysozyme, 1 mM PMSF, cOmplete™
1477 protease inhibitor cocktail (1 tablet per 50 mL), 10 mg/L DNase I, 20 mM MgSO₄) and
1478 lysed on ice using a tip sonicator with 5 cycles of 30 s "ON", 15 s "OFF". The lysate was
1479 clarified by centrifugation for 30 min at 20,000 g and 4°C, and the supernatant loaded on
1480 a StrepTrap HP column. Strep-tag-II-containing septin complexes were eluted with 50
1481 mM Tris-HCl pH 8, 300 mM KCl, 5 mM MgCl₂, and 2.5 mM desthiobiotin. The pooled
1482 fractions were then loaded to a HisTrap HP column, and His₆-tag-containing complexes
1483 eluted with 50 mM Tris-HCl at pH 8, 300 mM KCl, 5 mM MgCl₂, and 250 mM imidazole.
1484 Only the highest-concentration peak fractions were collected. Both affinity steps were
1485 performed on an ÄKTA pure protein purification system at 4°C (Cytiva). To remove
1486 imidazole, we either performed overnight dialysis or used a PD-10 column, also including
1487 DTT in this last step. The final elution buffer, in which septins are stored, was 50 mM Tris-
1488 HCl pH 8, 300 mM KCl, 5 mM MgCl₂, and 1 mM DTT. Protein concentration was assessed
1489 with absorbance measurements at 280 nm from the calculated extinction coefficients
1490 using ExPASy, and protein aliquots were flash-frozen in liquid nitrogen and stored at -
1491 80°C until further use.

1492 Chemicals used for recombinant septin complex production and purification are as
1493 follows. *E. coli* BL21(DE3) from Agilent (200131). Carbenicillin (C3416), spectinomycin
1494 (S4014), LB broth medium (L3022), LB agar (L2897), SOC medium (S1797) from Sigma.
1495 Terrific Broth from MP Biomedicals (091012017). IPTG (EU0008-C) and lysozyme (5933)
1496 from Euromedex. Imidazole from Fisher Scientific (Fisher Chemical I/0010/53). PMSF
1497 (78830), cOmplete™ Protease Inhibitor Cocktail Tablets (Roche, 11836145001), DNase
1498 I (Roche, 10104159001), *d*-Desthiobiotin (D1411), and DTT (D0632) from Sigma. HisTrap
1499 HP 1 mL columns (17524701) and StrepTrap HP 1 mL columns from Cytiva (28907546).
1500 20K MWCO Slide-A-Lyzer cassettes from Thermo Scientific (87735). PD-10 desalting
1501 columns from Cytiva (17085101).

1502

1503 **Sample preparation for fluorescence microscopy of *in vitro* reconstituted actin and**
1504 **septins.** To prepare flow cells, glass slides and coverslips were cleaned for 15 min in
1505 base-piranha solution (Milli-Q water, 30% ammonium hydroxide, 35% hydrogen peroxide
1506 at a 5:1:1 volume ratio), rinsed with Milli-Q water and stored in 0.1 M KOH up to one
1507 month. Right before assembling flow cells, slides and coverslips were rinsed with Milli-Q
1508 water and dried with synthetic air. Flow cells with ~10 μL channels were assembled by
1509 sandwiching ~2-mm-wide and ~2.5-cm-long strips of Parafilm between a cleaned glass
1510 slide and coverslip and melting on a hot plate at 120°C. The resulting chambers were

1511 passivated by incubating for 45 min with 1 M KOH, rinsing with actin polymerization buffer
1512 (5 mM Tris-HCl pH 8, 50 mM KCl, 1 mM MgCl₂, 0.2 mM Na₂ATP, 1 mM DTT), incubating
1513 for another 45 min with 0.2 mg/mL PLL-PEG, and rinsing with actin polymerization buffer.
1514 Flow cells were placed in a Petri-dish along with tissue paper soaked in water to prevent
1515 flow channels from drying during the incubation steps and until use.

1516 Lyophilized rabbit skeletal muscle G-actin was resuspended to 5 mg/mL (119 μM) in G-
1517 buffer (5 mM Tris-HCl pH 8, 0.2 mM Na₂ATP, 0.1 mM CaCl₂, 1 mM DTT), aliquots snap-
1518 frozen in liquid nitrogen and stored at -80°C. Frozen aliquots were thawed and
1519 centrifuged for 30 min at 120,000 g in a benchtop Beckman air-driven ultracentrifuge
1520 (Beckman Coulter Airfuge, 340401) to clear the solution from aggregates. Clarified G-
1521 actin was kept at 4°C and used within 3-4 weeks.

1522 For actin-septin reconstitution experiments, thawed septin aliquots were cleared for 15
1523 min at 120,000 g in a Beckman airfuge right before use. To polymerize G-actin in the
1524 presence of septins, we mixed G-actin, previously diluted with G-buffer to 5 μM, with
1525 septins, either nonfluorescent ones or msfGFP-labeled septins (at 20% msfGFP molar
1526 ratio for wild-type septins, and 100% GFP for SEPT2NC septins) to a final actin
1527 concentration of 1 μM and a final septin concentration of 0.3 μM, right before
1528 polymerization in actin polymerization buffer, additionally containing 1 mM Trolox, 2 mM
1529 protocatechuic acid (PCA), 0.1 μM protocatechuate 3,4-dioxygenase (PCD) and 0.1%
1530 w/v methylcellulose. To fluorescently label actin filaments, we polymerized G-actin in the
1531 presence of 1 μM Alexa Fluor 568-conjugated phalloidin.

1532 Actin-septin samples were prepared with a final volume of 10 μL, were loaded
1533 immediately into passivated flow channels upon mixing of the components to start
1534 polymerization, and flow channels were sealed with VALAP (1:1:1
1535 vasoline:lanoline:paraffin). The contributions of KCl and MgCl₂ from the septin elution
1536 buffer were taken into account to yield the same final composition of actin polymerization
1537 buffer. Actin-septin samples were incubated overnight at room temperature (RT) in the
1538 dark before observation. To polymerize septins in the absence of actin, we followed the
1539 same procedure as above, but replaced the G-actin solution with G-buffer. Septins were
1540 used at 20% msfGFP and 20% sfCherry2 molar ratio for wild-type septins and at 100%
1541 GFP for SEPT2NC septins.

1542 The sources and identifiers for proteins, materials and chemicals are as follows. Glass
1543 slides (26x76 mm) (AA00000102E01FST20) and glass coverslips (24x60 mm)
1544 (BB02400600A113FST0) from Thermo Scientific. Ammonium hydroxide solution
1545 (221228) and hydrogen peroxide solution (95299) from SIGMA. PLL-PEG from SuSoS
1546 AG (PLL(20)-g[3.5]-PEG(2)). Rabbit skeletal muscle G-actin from Cytoskeleton, Inc.
1547 (AKL99). Alexa Fluor 568-phalloidin from Thermo Scientific (A12380). Methylcellulose
1548 (M0512), Trolox (238813), protocatechuic acid (03930590), protocatechuate 3,4-
1549 dioxygenase (P8279) from Sigma.

1550

1551 **Confocal fluorescence microscopy of reconstituted actin-septins and image**
1552 **processing.** Reconstituted actin-septin assemblies were imaged on the same spinning
1553 disk microscope setup described for imaging cells using the same objective lens and
1554 camera. Images were acquired with an exposure time of 0.1 s. Actin-septin bundles were
1555 imaged close to the surface. Septin filament bundles were also found at the surface, but
1556 the clusters of interconnected filament bundles were observed floating in the bulk of the
1557 flow channels. To capture such clusters, z-stacks were acquired over 10-50 μm using a
1558 Δz interval of 0.5 μm . Images were processed with ImageJ/Fiji. Images of actin-septin
1559 bundles are from single planes. Images of septin filament bundles are from maximum-
1560 intensity z projections. The contrast of all images shown was adjusted post-acquisition so
1561 that both dim and bright structures are visible without saturation. All images use an
1562 inverted grayscale, with bright signals appearing black in a white background.

1563

1564 **Metal-induced energy transfer assays**

1565 U2OS cells were transfected with SEPT9_i3-mApple, SEPT9_i3-mApple-CAAX, or
1566 GAP43-mApple with FuGeneHD (Promega E2311). 16h post-transfection, cells were
1567 plated on glass coverslips (for obtaining reference lifetime measurements, see below)
1568 and on gold-coated glass coverslips, previously cleaned with 70% ethanol. Cells were left
1569 to attach and spread for 24h, then fixed for 15 min using 4% paraformaldehyde (Electron
1570 Microscopy Sciences 15714) in cytoskeleton buffer (10 mM MES – pH 6.1 with NaOH,
1571 150 mM NaCl, 5 mM EGTA, 5 mM glucose, 5 mM MgCl_2). The excess of cytosolic protein
1572 content was washed out with a permeabilization/blocking step (0,1% saponin, 1% BSA in
1573 PBS) for 1h at room temperature. Labelling of F-actin in U2OS cells was achieved with
1574 AlexaFluor568-phalloidin (Invitrogen A12380) at 165 nM in permeabilization/blocking
1575 solution for 1h. The samples were maintained in PBS until and throughout the
1576 measurements.

1577 Metal-induced energy transfer (MIET) was performed following the concept introduced by
1578 Enderlein and coworkers (Chizhik et al., 2014). Briefly, we measured the fluorescence
1579 lifetime of emitters in the vicinity of a 18 nm-thick gold film. From the calibration of the
1580 fluorescence lifetime dependence with the distance to the gold film (Chizhik et al., 2014),
1581 the distance between the fluorophore and the metal is recovered. The MIET calibration
1582 curve was computed using the MIET-GUI Matlab code developed by the Enderlein group
1583 (https://projects.gwdg.de/projects/miet/repository/raw/MIET_GUI.zip?rev=ZIP). For
1584 mApple, we used a peak emission wavelength at 610 nm and a quantum yield of 49%.
1585 For Alexa Fluor 568, the emission peak was 603 nm and the quantum yield 69%. Our
1586 calculation used the fluorescence lifetime of the dyes measured on a glass coverslip, in
1587 the absence of the metal layer, to account for the slight 0.1 ns lifetime change induced by
1588 the functionalization of the dye to septin, GAP43 or phalloidin. An isotropic orientation of
1589 the fluorophores is assumed (Chizhik et al., 2014).

1590 We used a gold film of 18 nm thickness deposited by electron-beam assisted evaporation
1591 of gold on a borosilicate glass coverslip (Bühler Syrus Pro 710). A 2 nm-thick chromium

1592 layer is used to promote the adhesion of gold on the glass coverslip. For the MIET
1593 calibration, the refractive indexes of the gold and chromium layers were taken from
1594 (Rosenblatt et al., 2020) and (Johnson and Christy, 1974), respectively while the
1595 refractive index of 1.52 for the borosilicate glass coverslip was provided by the supplier
1596 (D 263 M glass by Schott AG).

1597 The fluorescence lifetime measurements were performed with a home built confocal
1598 microscope with a 557 nm iChrome-TVIS laser (Toptica GmbH, pulse duration 3 ps, 40
1599 MHz repetition rate) and a Zeiss C-Apochromat 63x, 1.2 NA water immersion objective.
1600 The excitation power remained below 2 μ W on the sample to avoid photobleaching during
1601 the measurement. The fluorescence light was collected by the same microscope objective
1602 and filtered using a dichroic mirror (ZT 405/488/561/640rpc, Chroma), long-pass filter
1603 (ET570LP, Chroma) and bandpass filter (ET595/50m, Chroma). The confocal pinhole
1604 diameter was 50 μ m. The photon counting detection used an avalanche photodiode
1605 (MPD-5CTC, Picoquant) connected to a time correlated counting module (HydraHarp400,
1606 PicoQuant). The temporal resolution (full width at half maximum of the instrument
1607 response function) was measured to be 38 ps. The fluorescence lifetime histograms were
1608 fitted using SymPhoTime 64 software (PicoQuant GmbH) with a reconvolution taking into
1609 account the measured instrument response function. All the histograms were fitted using
1610 a biexponential function which provided a better fit to the intensity decay than a single
1611 exponential decay. About 20% of the total detected intensity corresponded to the short
1612 lifetime component (below 0.5 ns) which was not considered further for the analysis. The
1613 MIET distance measurements were taken on the long lifetime component which
1614 represented more than 80% of the total detected photons. The distribution of calculated
1615 distances from lifetime measurements for each condition is represented in box plots using
1616 GraphPad Prism (one data point per cell for each condition). The central mark indicates
1617 the median, and the bottom and top edges of the box indicate the 25th and 75th
1618 percentiles, respectively. The whiskers extend to the minimum and maximum values. The
1619 number of cells per condition is indicated in the respective legend. One-way ANOVA
1620 followed by a multiple comparison test was used for comparing the distributions. Data
1621 distribution was assumed to be normal but this was not formally tested.

1622

1623 **Supported lipid bilayer assays**

1624 **Small unilamellar vesicle formation.** We used three types of lipids, 1,2-dioleoyl-sn-
1625 glycerol-3-phospho-(1'-myo-inositol-4',5'-bisphosphate) (ammonium salt) (PI(4,5)P₂)
1626 (Sigma 850155P), 1,2-dioleoyl-sn-glycerol-3-phosphocholine (DOPC) (Sigma 850375C),
1627 and 1,2-dioleoyl-sn-glycerol-3-phosphoethanolamine-N-(Cyanine 5) (DOPE-Cy5) (Sigma
1628 810335C), all from Avanti Polar Lipids. The lipids were mixed in chloroform, or, in case
1629 PI(4,5)P₂ was present, in a 20:9:1 chloroform:methanol:water mixture in a glass vial. The
1630 organic solvent was then evaporated completely using a stream of N₂ followed by
1631 overnight incubation in a desiccator. The dried lipid film was resuspended in buffer to give
1632 a total lipid concentration of 0.25 mM. We used a sodium citrate buffer of pH 4.8 (50 mM

1633 citrate, made of equal molarity trisodium citrate and citric acid mixed in a 2:3 volume ratio,
1634 50 mM KCl, 0.1 mM ethylenediaminetetraacetic acid) in case PI(4,5)P₂ was present, and
1635 otherwise F-buffer of pH 7.4 (20 mM Tris-HCl, 2 mM MgCl₂, 50 mM KCl, 1 mM DTT). The
1636 lipids were dissolved by four cycles of 1 min vortexing and 5 min incubation. Finally, small
1637 unilamellar vesicles (SUVs) were obtained by sonicating the lipid solution using an
1638 Ultrasonic homogeniser series HD 2000.2 sonicator equipped with a BR30 cup resonator
1639 (Bandelin) at 10% amplitude for 30 minutes with pulses of 5s on and 5s off to avoid
1640 excessive heating.

1641 **Protein preparation.** Unlabelled septin octamers and SEPT2-msfGFP octamers were
1642 purified in house as previously reported (Iv et al., 2021). The protein was stored in aliquots
1643 in septin storage buffer (20 mM Tris HCl pH 7.4, 2 mM MgCl₂, 300 mM KCl, 1 mM DTT),
1644 at -80°C. Before each experiment, unlabelled and labelled septin octamers were mixed
1645 in a 9:1 molar ratio in septin storage buffer at a total concentration of 1800 nM. Lyophilized
1646 monomeric actin (G-actin) from rabbit skeletal muscle (Hypermol 8101-03) was
1647 resuspended following the manufacturer's instructions and dialyzed against G-buffer (5
1648 mM Tris-HCl pH 7.8, 0.1 mM CaCl₂, 0.2 mM ATP, and 1 mM DTT) to remove residual
1649 disaccharides from the freeze-drying process. Protein aggregates were removed by
1650 centrifugation at 148,000 x g for 1h and the supernatant was snap-frozen and stored in
1651 aliquots at -80°C. Fluorescently tagged G-actin was prepared by covalent modification
1652 with Alexa Fluor™ 594 Carboxylic Acid (Thermo Fisher Scientific 15461054) (Alvarado
1653 and Koenderink, 2015). Before experiments, G-actin aliquots were thawed, and any
1654 aggregates were removed by leaving the protein on ice for at least 2h and subsequently
1655 centrifuging at 148,000 x g for 20 min. Unlabelled and fluorescent actin were mixed in a
1656 9:1 molar ratio in G-buffer at a total G-actin concentration of 5 μM.

1657 **Sample preparation.** Supported lipid bilayers (SLB) were formed in custom-made flow
1658 channels made of nr. 1 Menzel coverslips (Thermo Fisher Scientific 11961988) and glass
1659 slides (Thermo Fisher Scientific 11879022). The coverslips and glass slides were first
1660 cleaned in base piranha solution (5% hydrogen peroxide, 5% ammonium hydroxide) at
1661 70°C for 10 minutes, extensively washed with Milli-Q water, and stored in Milli-Q water
1662 for a maximum of 5 days. Just before use, a coverslip and a slide were dried with a stream
1663 of N₂ gas. Flow channels were prepared by sandwiching 2x20 mm parafilm strips
1664 separated by ~3 mm between the glass slide and the coverslip. The parafilm was then
1665 melted by placing the chambers on a hot plate at 120°C and gently pressing on top with
1666 clean tweezers. After cooling down, an SUV solution (7-12 μL, depending on the distance
1667 between the parafilm strips) was pipetted into the channels and incubated in a humid
1668 chamber for at least 20 minutes to promote SUV rupture and SLB formation. Residual
1669 SUVs were removed by washing with 4 channel volumes of F-buffer for DOPC SLBs or
1670 with 2 channel volumes of sodium citrate buffer followed by 2 channel volumes of F-buffer
1671 for 5% PI(4,5)P₂ SLBs. DOPC SLBs contained 99.7% DOPC and 0.3% DOPE-Cy5;
1672 5%PIP₂ SLBs also contained 94.7% DOPC and 0.3% DOPE-Cy5.

1673 Septin octamers and actin were co-polymerized at room temperature in polymerization
1674 buffer (20 mM Tris-HCl pH 7.4, 2 mM MgCl₂, 50 mM KCl, 1 mM DTT, 0.5 mM ATP, 1 mM
1675 GTP) supplemented with 1 mM Trolox to suppress blinking, and an oxygen scavenging
1676 system composed of 1 mM protocatechuic acid and 0.05 μM of procatechuate 3,4-
1677 dioxygenase to minimize photobleaching. We first prepared a 5x master buffer (100 mM
1678 Tris HCl pH 7.4, 10 mM MgCl₂, 5 mM DTT, 2.5 mM ATP, 5 mM GTP, 5 mM Trolox and 5
1679 mM protocatechuic acid). To prepare the sample, we mixed the master buffer (5-fold
1680 dilution), 0.05 μM of procatechuate 3,4-dioxygenase, the G-actin mix (5-fold dilution to
1681 give a final concentration of 1 μM), and the septin mix (6-fold dilution, to give a final
1682 concentration of 300 nM), in that order. The mixture was either immediately added to the
1683 flow channels containing the SLBs and incubated for 1h in a humid environment, or first
1684 incubated in the tube for 1h to promote septin-actin bundle formation and then added to
1685 the flow channels using a cut pipette tip to minimize bundle disruption. For the sequential
1686 addition of septin octamers and actin, first septin octamers were diluted into the
1687 polymerization buffer, immediately added to the flow channels, and incubated for ~30
1688 minutes in a humid environment. Afterwards, the channels were washed with 1x channel
1689 volumes of F-buffer to remove unbound septin octamers; only 1x channel volume is used
1690 in this case to minimize disruption of the membrane-bound septin filament mesh. Finally,
1691 either pre-polymerized F-actin in polymerization buffer, or G-actin freshly added to
1692 polymerization buffer, was flushed into the flow channels with a cut tip to minimize actin
1693 filament disruption and incubated for 1h. The channels were then sealed with Dow
1694 Corning® high-vacuum silicone grease (Sigma Z273554) to avoid drying while imaging.
1695 **Image acquisition.** The samples were immediately imaged using a Nikon Ti2-E
1696 microscope complemented with a Gataca iLAS2 azimuthal TIRF illumination system. The
1697 sample was illuminated with 488-nm and 561-nm lasers (Gataca laser combiner iLAS2)
1698 to visualize the septin and the actin signals, respectively. The fluorescence signal was
1699 split with a Cairn Research Optosplit II ByPass containing a Chroma ZT 543 rdc dichroic
1700 mirror and filtered with either a 525/50 or a 600/50 chroma bandpass filter. The images
1701 were collected with a Nikon Apo TIRF 100x oil, NA 1.49 objective and recorded with an
1702 Andor iXon Ultra 897 EM-CCD camera using an exposure time of 50 ms. To check that
1703 the SLBs were uniform and free of defects, we examined DOPE-Cy5 distribution by
1704 illuminating with a 642-nm laser filtered with a 708/75 chroma bandpass filter and
1705 recorded using an exposure time of 20 ms. We checked SLB fluidity by fluorescence
1706 recovery after photobleaching of DOPE-Cy5.

1707

1708 **Atomic force microscopy**

1709 **Sample preparation.** Measurements were made on cells plated on Y shape-
1710 micropatterned substrates to minimize variability due to size and shape differences
1711 among cells (Rigato et al., 2015). 12-mm glass coverslips were coated with 0.1 mg/mL
1712 PLL-PEG (PLL(20)-g[3.5]-PEG(2), Susos) before being illuminated with a deep-UV lamp
1713 through a quartz-chrome photomask bearing the micropattern features (Front Range

1714 Photomask) designed using AutoCAD (Autodesk). We used Y-shaped micropatterns with
 1715 a spread area of $\sim 1500 \text{ mm}^2$. Micropatterned coverslips were then incubated with 25
 1716 $\mu\text{g/mL}$ fibronectin and 5 $\mu\text{g/mL}$ fibrinogen-GFP, the latter for visualizing micropatterns.
 1717 Wild type U2OS cells treated with siLacZ siRNA (6mer+8mer), with SEPT9 siRNA (6mer),
 1718 and U2OS cells treated with SEPT7 siRNA and also transfected with msfGFP-
 1719 SEPT7Gmut2 (8mer) were seeded on fibronectin-coated micropatterns 48 h post-
 1720 electroporation. Cells were incubated for 5-7 h to attach and spread adopting a triangular
 1721 shape. The expression of msfGFP-SEPT7Gmut2 for the 8mer condition was confirmed
 1722 through the detection of fluorescence in each measured cell.

1723 **Force Spectroscopy experiments and data analysis.** Atomic force microscopy-force
 1724 spectroscopy (AFM-FS) was performed on the dorsal perinuclear region of individual cells
 1725 at room temperature. We used a MLCT-Bio-DC (D) cantilever featuring a 4-sided regular
 1726 pyramid with a semi-open angle of 35° . The spring constant of the cantilevers was
 1727 determined in air using the Sader method (Sader et al., 2012) and the optical lever
 1728 sensitivity from the thermal spectrum in liquid (Sumbul et al., 2020). Force-distance
 1729 curves were acquired applying a maximum force of 0.8 nN with a ramp range of 5 μm , at
 1730 the same approach and retract velocity of 5 $\mu\text{m/s}$ on a Nanowizard 4 AFM microscope
 1731 (JPK-Bruker). The indentation depth was on the order of 1 μm . 31 cells, 29 cells and 23
 1732 cells were probed for the 6mer+8mer, 6mer and 8mer condition, respectively. For each
 1733 cell, about 15-30 force curves were acquired across 3 different contact points, resulting
 1734 in a total of 576, 630 and 501 force curves for the 6mer+8mer, 6mer and 8mer condition,
 1735 respectively. To extract the cell viscoelastic properties, we fitted the Ting numerical
 1736 viscoelastic model for a 4-sided regular pyramidal tip of semi-open angle (θ) to the
 1737 experimental force-distance curves (Bilodeau, 1992; Efremov et al., 2017):
 1738

$$1739 \quad F(t, \delta(t)) = \begin{cases} \frac{3 \tan \theta}{4(1 - \nu^2)} \int_0^t E(t - \tau) \frac{\partial \delta^2}{\partial \tau} d\tau, 0 \leq t \leq t_m \\ \frac{3 \tan \theta}{4(1 - \nu^2)} \int_0^{t_1} E(t - \tau) \frac{\partial \delta^2}{\partial \tau} d\tau, t_m \leq t \leq t_{ind} \end{cases}$$

1740
 1741 where F is the applied force; δ is the indentation; t is the time since initial contact, t_m is the
 1742 duration of approach trace, t_{ind} is the duration of complete indentation cycle, and t_1
 1743 determined by solving the equation
 1744

$$1745 \quad \int_{t_1(t)}^t E(t - \tau) \frac{\partial \delta(t)}{\partial \tau} d\tau = 0$$

1746
 1747 We assumed that the time-dependent Young's modulus followed a power law
 1748 relationship:

$$1749 \quad E(t) = E_0 \left(\frac{t}{t_0} \right)^{-\beta}$$

1750 where E_0 is the elastic modulus at time t_0 , β is the fluidity of the cell and t_0 is the reference
1751 time, arbitrarily assumed 1s. A viscous drag force (F_d) proportional to the trace velocity
1752 (v) was also added to the force traces using a precalibrated value of the viscous drag
1753 coefficient ($b= 5 \text{ pN}\cdot\text{s}/\mu\text{m}$), $F_d=b\cdot v$.

1754 The values of $\log_{10}(E_0)$ and β extracted from each force measurement were pooled by
1755 cell and then averaged. The data was reproduced in 3 independent experiments and their
1756 distribution represented in box plots using GraphPad Prism (one data point per cell for
1757 each condition). The central mark indicates the median, and the bottom and top edges of
1758 the box indicate the 25th and 75th percentiles, respectively. The whiskers extend to the
1759 minimum and maximum values. The number of cells per condition is indicated in the
1760 respective legend. E_0 values (in Pa) were plotted on a log scale. One-way ANOVA
1761 followed by a multiple comparison test was used for comparing the distributions of E_0
1762 values using the $\log_{10}(E_0)$ values, given the log-normal distribution of E_0 .

1763

1764 **Single particle tracking Photo-Activated Localization Microscopy (sptPALM)**

1765 **Cell culture.** Mouse embryonic fibroblasts were cultured in DMEM (Gibco 10313-021)
1766 with 10% fetal calf serum (FCS, Eurobio scientific CVFSVF00-01). Transient transfections
1767 of plasmids were performed 2 days before experiments using the Amaxa nucleofector
1768 (Lonza VPD-1004). The cells were detached with trypsin/EDTA, the trypsin was
1769 inactivated using DMEM with 10% FCS, and the cells were washed and suspended in
1770 serum-free Ringer solution (150 mM NaCl, 5 mM KCl, 2 mM CaCl_2 , 2 mM MgCl_2 , 10 mM
1771 HEPES-Na pH 7.4, 2 g/L glucose), then incubated for 30 min in Ringer solution before
1772 plating on fibronectin-coated glass coverslips (human plasma fibronectin at 10 $\mu\text{g}/\text{ml}$,
1773 Roche 10838039001).

1774 **Plasmids.** SEPT9_i3-mEos3.2 and SEPT9_i3-mEos3.2-CAAX were cloned in a pCMV
1775 plasmid backbone with seamless cloning into a NheI/BamHI linearized vector (primers in
1776 Table S1). EGFP-human β -actin was provided by A. Matus (Friedrich Miescher Institute
1777 for Biomedical Research, Switzerland). The mEos2-actin construct was generated from
1778 EGFP-actin as described in (Rossier et al., 2012). EYFP-human paxillin (isoform alpha)
1779 was used as described in (Rossier et al., 2012).

1780 **Optical setup and image acquisition.** sptPALM acquisitions were steered by
1781 MetaMorph software (Molecular Devices) with an inverted motorized microscope (Nikon
1782 Ti) equipped with a temperature control system (The Cube, The Box, Life Imaging
1783 Services), a Nikon CFI Apo TIRF 100x oil, NA 1.49 objective and a Perfect Focus System,
1784 allowing long acquisition in TIRF illumination mode.

1785 Imaging was performed at least 3 hours after seeding the cells on fibronectin-coated
1786 coverslips mounted in a Ludin chamber (Life Imaging Services). For photoactivation
1787 localization microscopy, cells expressing mEos2 and mEos3.2 tagged constructs were
1788 photoactivated using a 405 nm laser (Omicron) and the resulting photoconverted single
1789 molecule fluorescence was excited with a 561 nm laser (Cobolt Jive™). Both lasers
1790 illuminated the sample simultaneously. Their respective power was adjusted to keep the

1791 number of the stochastically activated molecules constant and well separated during the
 1792 acquisition. Fluorescence was collected by the combination of a dichroic and emission
 1793 filters (D101-R561 and F39-617 respectively, Chroma) and a sensitive EMCCD (electron-
 1794 multiplying charge-coupled device, Evolve, Photometric). The acquisition was performed
 1795 in streaming mode at 50 Hz. Either EYFP-paxillin or GFP-actin were imaged using a
 1796 conventional GFP filter cube (ET470/40, T495LPXR, ET525/50, Chroma). Using this filter
 1797 cube does not allow spectral separation of the unconverted pool of mEos from the GFP
 1798 fluorescent signal. However, with all of the constructs used, whether the mEos signal was
 1799 highly or poorly enriched in FAs, we were still able to detect FAs.

1800 **Single molecule segmentation and tracking.** A typical sptPALM experiment leads to a
 1801 set of at least 4000 images per cell, analyzed in order to extract molecule localization and
 1802 dynamics. Single molecule fluorescent spots were localized and tracked over time using
 1803 a combination of wavelet segmentation and simulated annealing algorithms (Izeddin et
 1804 al., 2012; Racine et al., 2006; Racine et al., 2007). Under the experimental conditions
 1805 described above, the resolution of the system was quantified to 59 nm (Full Width at Half
 1806 Maximum, FWHM). This spatial resolution depends on the image signal to noise ratio and
 1807 the segmentation algorithm (Cheezum et al., 2001) and was determined using fixed
 1808 mEos2 samples. We analyzed 130 2D distributions of single molecule positions belonging
 1809 to long trajectories (>50 frames) by bi-dimensional Gaussian fitting, the resolution being
 1810 determined as $2.3 s_{xy}$, where s_{xy} is the pointing accuracy.

1811 For the trajectory analysis, FAs ROIs were identified manually from EYFP-paxillin or GFP-
 1812 actin images. The corresponding binary mask was used to sort single-molecule data
 1813 analyses to specific regions. We analyzed trajectories lasting at least 260 ms (≥ 13 points)
 1814 with a custom Matlab routine analyzing the mean squared displacement (MSD), which
 1815 describes the diffusion properties of a molecule, computed as (Eq. 1):

$$1816$$

$$1817 \text{MSD}(t = n \cdot \Delta t) = \frac{\sum_{i=1}^{N-n} (x_{i+n} - x_i)^2 + (y_{i+n} - y_i)^2}{N - n} \quad \text{Eq. 1}$$

$$1818$$

$$1819$$

1820 where x_i and y_i are the coordinates of the label position at time $i \times \Delta t$. We defined the
 1821 measured diffusion coefficient D as the slope of the affine regression line fitted to the $n=1$
 1822 to 4 values of the $\text{MSD}(n \times \Delta t)$. The MSD was computed then fitted on a duration equal
 1823 to 80% (minimum of 10 points, 200 ms) of the whole stretch by (Eq. 2):

$$1824$$

$$1825 \text{MSD}(t) = \frac{4r_{\text{conf}}^2}{3} (1 - e^{-t/\tau}) \quad \text{Eq. 2}$$

$$1826$$

1827 where r_{conf} is the measured confinement radius and τ the time constant $\tau = (r_{\text{conf}}^2 / 3D_{\text{conf}})$.
 1828 To reduce the inaccuracy of the MSD fit due to downsampling for larger time intervals,
 1829 we used a weighted fit. Trajectories were sorted in 3 groups: immobile, confined diffusion

1830 and free diffusion. Immobile trajectories were defined as trajectories with $D < 0.011 \mu\text{m}^2 \cdot \text{s}^{-1}$,
1831 corresponding to molecules which explored an area inferior to the one defined by the
1832 image spatial resolution $\sim (0.05 \mu\text{m})^2$ during the time used to fit the initial slope of the MSD
1833 (Rossier et al., 2012) (4 points, 80 ms): $D_{\text{threshold}} = (0.059 \mu\text{m})^2 / (4 \times 4 \times 0.02 \text{s}) \sim 0.011 \mu\text{m}^2 \cdot \text{s}^{-1}$.
1834 To separate trajectories displaying free diffusion from confined diffusion, we used the time
1835 constant calculated τ for each trajectory. Confined and free diffusion events were defined
1836 as trajectories with a time constant respectively inferior and superior to half the time
1837 interval used to compute the MSD (100 ms). Statistical significance tests were prepared
1838 using GraphPad Prism.

1839

1840 **Modeling of human septin complexes**

1841 Models of full-length human septin complexes were built for analyzing and interpreting
1842 split-GFP experiments. The septin GTP-binding domains (GBDs) used as templates for
1843 the SEPT2, 6 and 7 models using SWISS-MODEL homology modeling software
1844 (Waterhouse et al., 2018) were from PDB 7M6J (Leonardo et al., 2021), the most
1845 complete human septin hexamer structure to date, which includes $\alpha 0$ helices for SEPT6
1846 and 7. As solved in its integrity, the SEPT6 GBD remained unchanged and was used as
1847 is. The SEPT7 GBD structure was completed using SWISS-MODEL. As the use of the
1848 SEPT2 GBD from 7M6J for modeling SEPT2 led to clashes in the modeled SEPT2-
1849 SEPT2 NC interface, the SEPT2 GBD subunit was modeled using the SEPT7 GBD
1850 structure from 7M6J as a template. The lack of structural information for the short N-
1851 terminal extensions of SEPT2, 6, and 7 prompted us to model them as disordered
1852 segments using Phyre2 (Kelley et al., 2015). The C-terminal domains of SEPT2, 6 and 7
1853 were modeled with CCFold (Guzenko and Strelkov, 2018) for the coiled-coil (CC) parts
1854 and Phyre2 for the flexible parts, as detailed in (Iv et al., 2021). The homodimeric parallel
1855 SEPT2CC was used unaltered with respect to (Iv et al., 2021). The previously modeled
1856 SEPT6 and 7 helices in the SEPT6-SEPT7 parallel coiled-coil in (Iv et al., 2021) were
1857 repositioned slightly after comparison with the only parallel septin CC structure to date
1858 (PDB 6WCU) (Leonardo et al., 2021). GBDs, N- and C-terminal extensions were then
1859 combined with PyMOL open-source software. When necessary, the disordered segments
1860 were manually modified to avoid steric clashes and to adjust distances. The SEPT9_i3
1861 model used was the one built for (Iv et al., 2021) and included already N- and C-terminal
1862 extensions. Hexameric SEPT2-SEPT6-SEPT7-SEPT7-SEPT6-SEPT2 and octameric
1863 SEPT2-SEPT6-SEPT7-SEPT9-SEPT9-SEPT7-SEPT6-SEPT2 complexes were built by
1864 fitting the modeled structures to the hexamer from the PDB 7M6J.

1865 To analyze and interpret the split-GFP experiments, the entire constructs used in the
1866 assays, including $\beta 10$ - and $\beta 11$ -tagged septins and the reconstituted GFP, were modeled.
1867 To this aim, the split GFP structure (PDB 4KF5) was added to the modeled septin
1868 complexes. The flexible linkers linking the reconstituted GFP to the septin of interest were
1869 built manually using PyMOL; their straight-ish appearance in the models is due to the
1870 polypeptide chains being built as linear structures. To mimic paired septin filaments with

1871 narrow spacing (Leonardo et al., 2021), mediated by homodimeric SEPT2 antiparallel
1872 CCs (Fig. 5H), septin complexes were duplicated and placed parallel to each other with
1873 a gap of ~5 nm. The bent conformation of the septin was built by rotating the CC domain
1874 manually by 90 degrees relative to the GBD. The helices within the homodimeric SEPT2
1875 antiparallel CC were positioned using the antiparallel SEPT4CC structure from PDB
1876 6WB3 as a reference (Leonardo et al., 2021). All manual interventions were realized using
1877 PyMOL.

1878
1879 **Statistics and reproducibility.** The distributions of measurements, or of phenotypes in
1880 the case of septin mutant characterization, are represented with GraphPad Prism using
1881 box plots, violin plots and scatter dot plots as indicated in the respective methods sections
1882 and legends. Bars, error bars (SD or SEM) and box plot features are as indicated in the
1883 respective figure legends. The number of measurements in each plot and the numbers of
1884 experiments are indicated in the respective figure legend or methods. Statistical
1885 significance tests were performed with with GraphPad Prism. The tests applied and the
1886 obtained P values are mentioned in the respective figure legend. Experiments were
1887 repeated at least three times independently to ensure reproducibility. Experiments from
1888 Fig. 1F; Fig. 2C,D; Fig. 10C-F; Fig 10H; Fig. S3B,C; Fig. S5I-K were performed twice.
1889 Experiments from Fig. 10G,I were performed once. No data were excluded from the
1890 analyses.

1891
1892 **Data availability.** All data supporting the findings of this study are available within the
1893 article and its supporting information files. The source datasets generated and analyzed
1894 during the current study are available from the corresponding author on reasonable
1895 request.

1896
1897 **Code availability.** The source codes for the custom-generated Matlab codes for
1898 measurements of fiber diameter (FWHM) and length, and for numerical simulations of
1899 expected fiber diameter (FWHM) from SIM images has been deposited to Github. The
1900 respective links are mentioned in the relevant methods sections.

1901
1902 **Supplemental material**
1903 Fig. S1 shows examples of SEPT2, SEPT7 and SEPT9 distribution in cells from
1904 immunostainings and live imaging of GFP fusions. Fig. S2 contains details on the design
1905 of the tripartite split-GFP complementation assay for probing septin organization. Fig. S3
1906 depicts the mutants used in this study, as well as examples of cell-free reconstitution of
1907 actin-septin assembly. Fig. S4 contains data that support that all septins on microtubules
1908 organize as octamer-based filaments. Fig. S5 contains data that support that septin
1909 filaments can mediate actin-membrane anchoring in the absence of myosin-II.

1910
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1954

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Supplementary Figure legends

Figure S1. **SEPT2, SEPT7 and SEPT9 distribution on different types of stress fibers in U2OS cells.** **(A)** Representative confocal micrographs of SEPT2 immunostained cells (i-iii) and cells expressing SEPT2-msfGFP (iv-v). SEPT2 immunostained cells are co-stained for F-actin (phalloidin) and the FA protein paxillin. Examples show SEPT2 localizing (i) to peripheral (a) and ventral (b,c) SFs and excluded from focal adhesions (FA) (c), (ii) to peripheral (a) and perinuclear actin caps (b), (iii) to transverse arcs (b) and excluded from dorsal SFs (a,b), (iv) to transverse arcs (a,b) and excluded from dorsal SFs (a), and (v) showing a diffuse cytosolic phenotype. **(B)** Representative confocal micrographs of SEPT9 immunostained cells (i-iii) and cells expressing SEPT9_i3-msfGFP (iv-v). SEPT9 immunostained cells are co-stained for F-actin (phalloidin) and the FA protein paxillin. Examples show SEPT9 localizing (i) to peripheral (a) and ventral (b,c) SFs and excluded from focal adhesions (FA) (c), (ii) to perinuclear actin caps (a,b), (iii) to transverse arcs (a) and ventral SFs (b), (iv) to transverse arcs (a) and excluded from dorsal SFs (a) and to ventral SFs (b), and (v) to peripheral (a) and perinuclear actin caps (b). **(C-D)** Representative confocal micrographs of SEPT7 immunostainings showing SEPT7 localizing to ventral actin nodes. Cells are co-stained for F-actin (phalloidin) and non-muscle myosin heavy chain IIA (NMIIA) (C) or α -actinin (D). Yellow arrowheads point to two actin nodes in each example. Scale bars in large fields of views, 10 μ m. Scale bars in insets, 2 μ m (A and B) and 5 μ m (C,D). Related to Fig. 1B.

Figure S2. **Design of the tripartite split-GFP complementation assay for probing septin organization.** **(A)** Sequences of the β -10 and β 11-tags used for all split assays and of the linker sequences tested in screening experiments (B,C); 14-residue linkers were used throughout this study. **(B)** Schematic of N- and C-terminal β -10 and β 11-tag septin fusions tested in screening experiments (C) using short or long linkers (A). **(C)** Schematic of β -10 and β 11-septin fusion combinations for screening tripartite split GFP complementation. Combinations with the same pattern were considered to be equivalent (for example, SEPT2-14- β 10 / β 11-14-SEPT2 and SEPT2-14- β 11 / β 10-14-SEPT2). The combinations in green are the ones tested experimentally. **(D)** Schematic of the pTRIP TRE Bi vector bearing a bidirectional tetracycline response element (TRE) promoter for the doxycycline-inducible co-expression of β 10- and β 11-tagged septins. An IRES-TagBFP cassette was used for monitoring septin expression. Restriction sites used for subcloning are indicated (see methods for details). **(E)** Left, Western blots of U2OS-Tet-On-GFP1-9 cell line lysates probed with anti-SEPT7, anti- β 10 and anti- α -tubulin antibodies upon treatment with siRNAs targeting LacZ (siCtrl), SEPT7 (siSEPT7), and targeting SEPT7 while co-expressing wild-type β 10- and β 11-SEPT7 (WT), β 10- and β 11-SEPT7Gmut1 (Gmut1), and β 10- and β 11-SEPT7Gmut2 (Gmut2). Yellow and orange arrowheads point to bands correspond to β 10- and β 11-fusions. The SEPT7 and β 10 blots are also shown saturated on purpose for displaying weaker bands. Molecular weight

markers are shown on the left. Middle, Western blots of U2OS-Tet-On-GFP1-9 cell line lysates probed with anti-SEPT2, anti- β 10, anti- β 11 and anti- α -tubulin antibodies upon treatment with siRNAs targeting LacZ (siCtrl), SEPT2 (siSEPT2), and targeting SEPT2 while co-expressing wild-type SEPT2- β 10 and - β 11 (WT) or SEPT2NCmut- β 10 and - β 11 (NCmut). Yellow and orange arrowheads point to bands correspond to β 10- and β 11-fusions. The SEPT2 blot is also shown saturated on purpose for displaying weaker bands. Right, Western blots of U2OS-Tet-On-GFP1-9 cell line lysates probed with anti-SEPT9, anti- β 10, anti- β 11 and anti- α -tubulin antibodies upon treatment with siRNAs targeting LacZ (siCtrl), SEPT9 (siSEPT9), and targeting SEPT9 while co-expressing wild-type SEPT9- β 10 and - β 11 (WT) or SEPT9NCmut- β 10 and - β 11 (NCmut) for both SEPT9_i1 and SEPT9_i3. Yellow and orange arrowheads point to bands correspond to β 10- and β 11-fusions. The SEPT9 blot is also shown saturated on purpose for displaying weaker bands. Asterisks point to SEPT9 degradation products. **(F)** Western blot of U2OS cell lysates probed with anti-SEPT2 and anti- α -tubulin antibodies upon treatment with siRNAs targeting LacZ (siCtrl) or SEPT2 (siSEPT2). Molecular weight markers are shown on the left. Bottom, respective quantification of SEPT2 protein levels (mean+SD). Mean values (normalized to 1 for siCtrl) are from 3 independent siCtrl and 9 independent siSEPT2 treatments. SEPT2 was knocked down on average by 92%. **(G)** Same as (F) for SEPT9. The asterisk points to a SEPT9 degradation product. Mean values (normalized to 1 for siCtrl) are from 3 independent siCtrl and 3 independent siSEPT9 treatments. SEPT9 was knocked down on average by 92%. **(H)** Same as (F) for SEPT7. Mean values (normalized to 1 for siCtrl) are from 3 independent siCtrl and 12 independent siSEPT7 treatments. SEPT7 was knocked down on average by 86%.

Figure S3. Septin interface mutants used in this study and cell-free reconstitution of septin assembly. **(A)** Left, Top, conserved residues in the SEPT2-SEPT2 NC interface are shown in the crystal structure of human SEPT2 homodimers (PDB 2QA5) (Sirajuddin et al., 2007). The backbone structure is displayed as a cartoon representation in PyMOL, with critical residues represented as sticks (deep blue and red for nitrogen and oxygen atoms, respectively). Residues F20 from the hook-loop of one SEPT2 subunit (orange) interact with the hydrophobic cleft formed by V86, I88, V93, and I281 of the adjacent SEPT2 subunit (blue). The importance of this phenylalanine in anchoring the α 0 helix at the NC interface was emphasized only recently (Cavini et al., 2021). The blue subunit's surface representation highlights the complementary of shape between the two SEPT2 subunits in this interface. The interaction between the α 0 helices of each subunit is also stabilized via a hydrophobic interaction between their respective V27. Left, Middle, sequence alignment of the regions including the residues shown in the NC interface structure for SEPT2 and SEPT9_i3. The structural elements (α 0, β 2, α 5) related to these residues are underlined and shown above the sequences. The consensus symbols are from ClustalW alignments of all human septins (*, fully conserved residue; colon, conservation between residues with strongly similar physicochemical properties; period,

conservation between residues with weakly similar physicochemical properties). We note that the residues described above are strictly or physicochemically conserved (except for V86), highlighting their importance in stabilizing the SEPT2-SEPT2 NC interface. Left, Bottom, NC interface mutants used in this study. A mutation of F20D/I263D is expected to destabilize the hydrophobic pocket depicted above, whereas a V27D/M270D is expected to destabilize the α 0 helices interface. Importantly, a strictly conserved aspartate (SEPT2 E90, corresponding to SEPT6 E90 and SEPT7 E102 which are well defined in the cryo-EM structure of the SEPT6-SEPT7 NC interface (Mendonca et al., 2021)) in the loop connecting β 2 and β 3 is pointing to the hydrophobic cleft where the phenylalanine resides. The F20D mutation is thus expected to result in a repulsion between the aspartate and glutamate and contribute further to the destabilization of the NC interface. Right, Top, conserved residues in the SEPT7-SEPT7 G interface are shown in the crystal structure of human SEPT7 homodimers (PDB 6N0B) (Brognara et al., 2019). The backbone structure is displayed as a cartoon representation in PyMOL, with critical residues represented as sticks (deep blue and red for nitrogen and oxygen atoms, respectively). Residues W269 of one SEPT7 subunit (yellow) interact with residues W269, A272 and H279 in the adjacent SEPT7 subunit (green) (Sirajuddin et al., 2007; Zent et al., 2011). W269 from adjacent subunits interact via a water molecule bridge through hydrogen bonds. In addition, each W269 is engaged in π - π interactions with H279 and CH- π interactions with A272 of the opposite subunit. Right, Middle, sequence alignment of the regions including the residues shown in the G interface structure for SEPT7 and SEPT9_i3. The structural elements (β 7, β 8) related to these residues are underlined and shown above the sequences. The consensus symbols are from ClustalW alignments of all human septins (*, fully conserved residue; colon, conservation between residues with strongly similar physicochemical properties). Notice that W269 and H279 are both strictly conserved, showing their importance in stabilizing this interface. Right, Bottom, G interface mutants that were used in this study. The presence of both mutations W269A and H279D in SEPT7 and SEPT9 is expected to destabilize the SEPT7-SEPT7 and SEPT7-SEPT9 G-interfaces. The loss of the aromatic cycle properties in the mutant W269A does not allow the abovementioned critical interactions mediated by the wild-type Trp. W269A is expected to destabilize H279 and potentially change its orientation. In addition, the much smaller size of the alanine will poorly mimic the hydrophobic interaction between W269 and H279, weakening the G-interface. Note that W269 is in the vicinity of Tyr267 of the same subunit. This tyrosine interacts with the nucleotide buried within the G-interface. Consequently, any mutations destabilizing W269 could dramatically destabilize the overall G-interface because of a domino effect. Similarly, H279D is expected to preclude hydrophobic interactions with W269 and thus destabilize the latter. The single mutation H279D in SEPT7 is expected to destabilize the SEPT7-SEPT7 G-interface when present in both SEPT7 subunits, but not the SEPT7-SEPT9 interface with wild-type SEPT9. **(B)** Representative spinning disk fluorescence images of septin filament assembly upon polymerization of hexamers in solution at the indicated final protomer

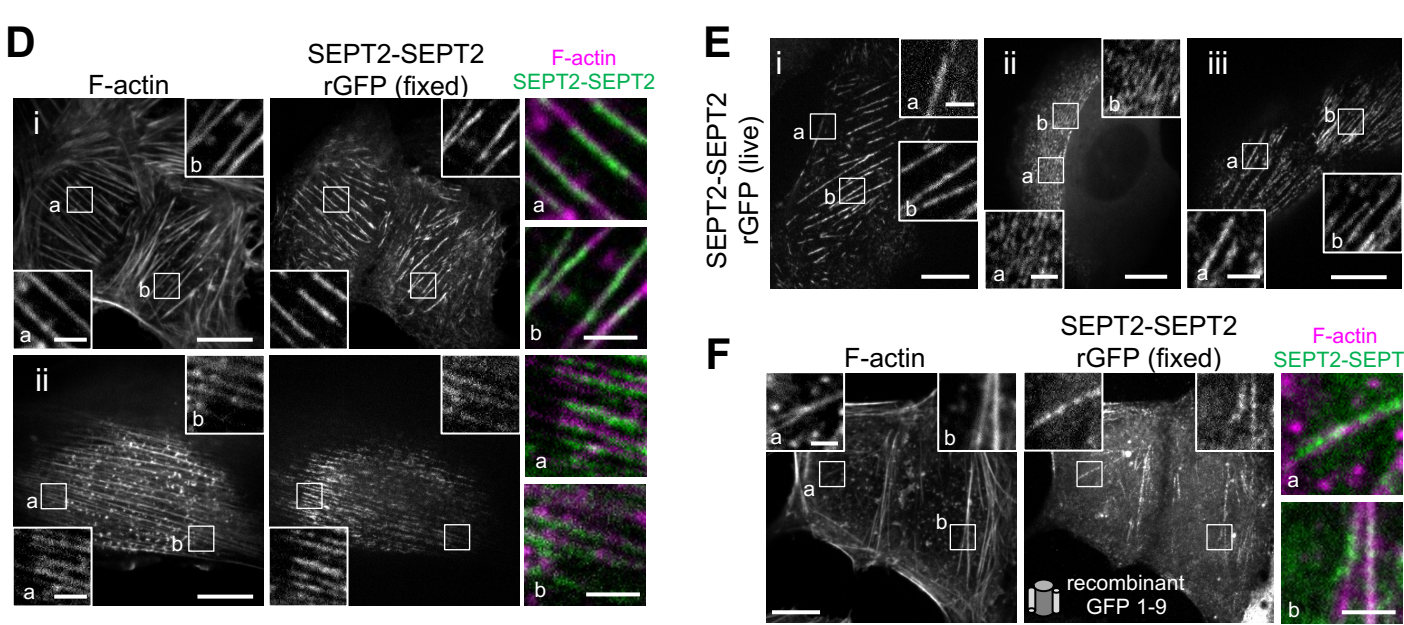
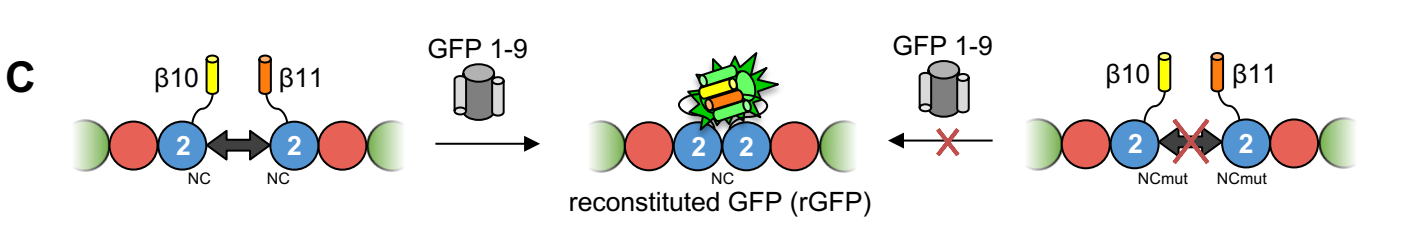
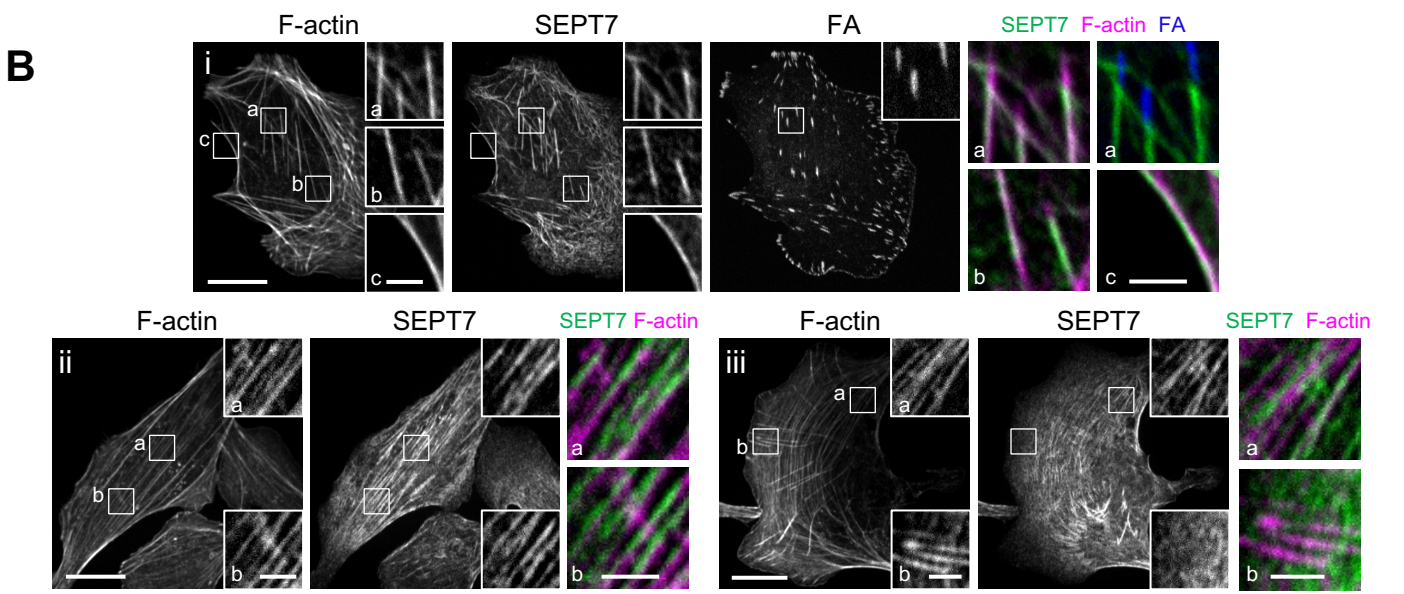
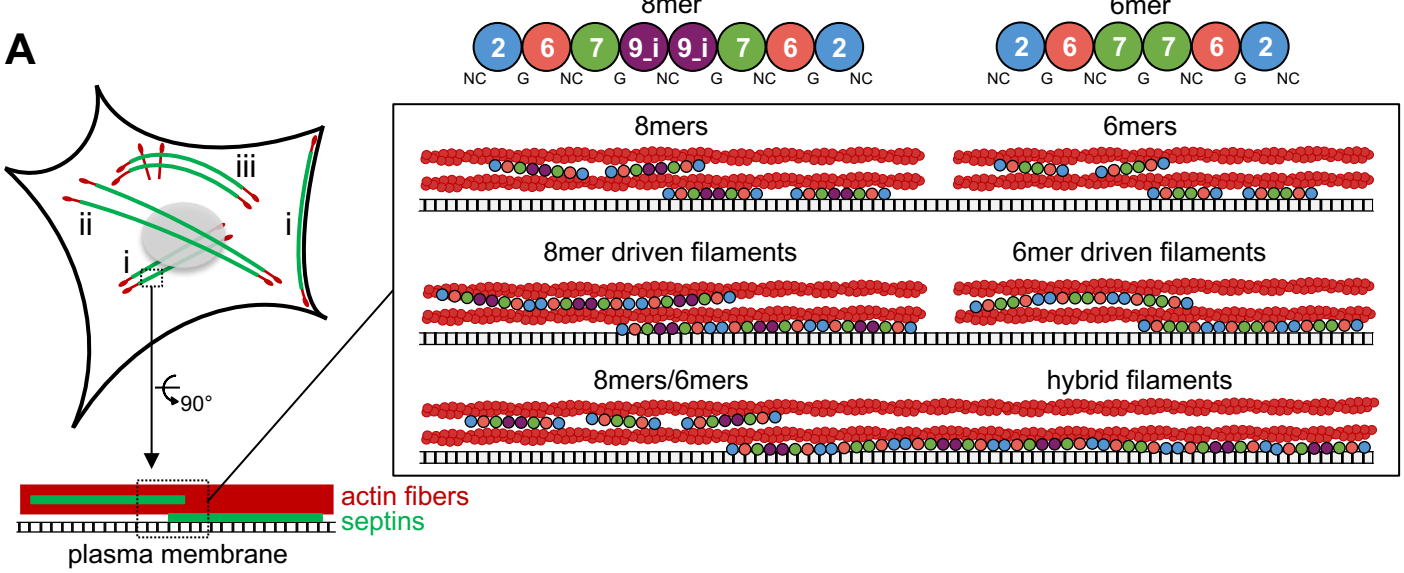
concentration. Protomers contained either wild-type SEPT2 (top panel) or SEPT2NCmut (bottom panel). Images use an inverted grayscale. Related to Fig. 2C. **(C)** Representative spinning disk fluorescence images of reconstituted actin filaments, polymerizing in the presence of septin hexamers in solution. Protomers contained either wild-type SEPT2 or SEPT2NCmut. Actin filaments are visualized with AlexaFluor568-conjugated phalloidin, and septins with SEPT2-msfGFP. One example of large fields of view are shown for each condition, depicting cross-linking of actin filaments; only actin labeling is shown. The image for actin in the presence of wild-type hexamers is contrast-enhanced on purpose in order to saturate the actin bundles so that weaker-intensity single actin filaments are also visible. Insets on the bottom show higher magnifications of selected regions of interest on the top (dashed squares in red). Two regions of interest (a,b for wild-type SEPT2 and c,d for SEPT2NCmut) are shown in each case, depicting both the actin and septin signals. Scale bars in all large fields of views, 10 μm . Scale bars in all insets, 5 μm . **(D)** Representative spinning disk fluorescence images of septin filament assembly upon co-polymerization of hexamers containing SEPT2-msfGFP and octamers-9_i3 containing SEPT2-sfCherry2 at the indicated final protomer concentration. Images use an inverted grayscale. Scale bars in all large fields of views, 10 μm .

Figure S4. All septins on microtubules organize as octamer-based filaments. (A) Violin plots depicting the distribution of diffuse cytosolic (red datapoints) vs. non-diffuse (green datapoints) phenotypes as a function of the intensity of the msfGFP signal in cells expressing wild-type SEPT9_i1-msfGFP or SEPT9_i1NCmut-msfGFP. Data points are from a total of 90 cells each for wild-type and mutant SEPT9 distributed among the two phenotypes. **(B)** Violin plots depicting the distribution of diffuse cytosolic (red datapoints) vs. non-diffuse (green datapoints) phenotypes as a function of the intensity of the rGFP signal in GFP1-9 cells co-expressing wild-type SEPT9_i1- β 10 and - β 11 or SEPT9_i1NCmut- β 10 and - β 11. Data points are from a total of 40 cells each for wild-type and mutant SEPT9 distributed among the two phenotypes. **(C)** Representative confocal micrograph of SEPT9_i1-SEPT9_i1 rGFP distribution in fixed cells co-stained for microtubules (α -tubulin). **(D)** Representative example of a GFP1-9 cell co-expressing β 11-SEPT7 and SEPT9_i1- β 10 and co-stained for α -tubulin, with rGFP localizing to microtubules. **(E)** Representative example of GFP1-9 cell co-expressing β 11-SEPT7Gmut1 and SEPT9_i1Gmut- β 10 co-stained for α -tubulin showing a diffuse cytosolic phenotype. **(F)** Representative confocal micrograph of SEPT2-SEPT2 rGFP distribution in a live cell co-expressing mCherry-SEPT9_i1 (not shown) and labeled for microtubules (SiR-tubulin). **(G)** Representative example of a cell (bottom left) co-expressing msfGFP-SEPT7 and mCherry1-SEPT9_i1 and labeled for microtubules (SiR-tubulin) showing msfGFP-SEPT7 localizing to microtubules (b). A cell expressing only msfGFP-SEPT7 (top right) shows msfGFP-SEPT7 localizing to ectopic bundles not co-localizing with microtubules (a). Scale bars in large fields of views, 10 μm . Scale bars in insets, 2 μm . Related to Fig. 5E. **(H)** Representative confocal micrograph of SEPT7-

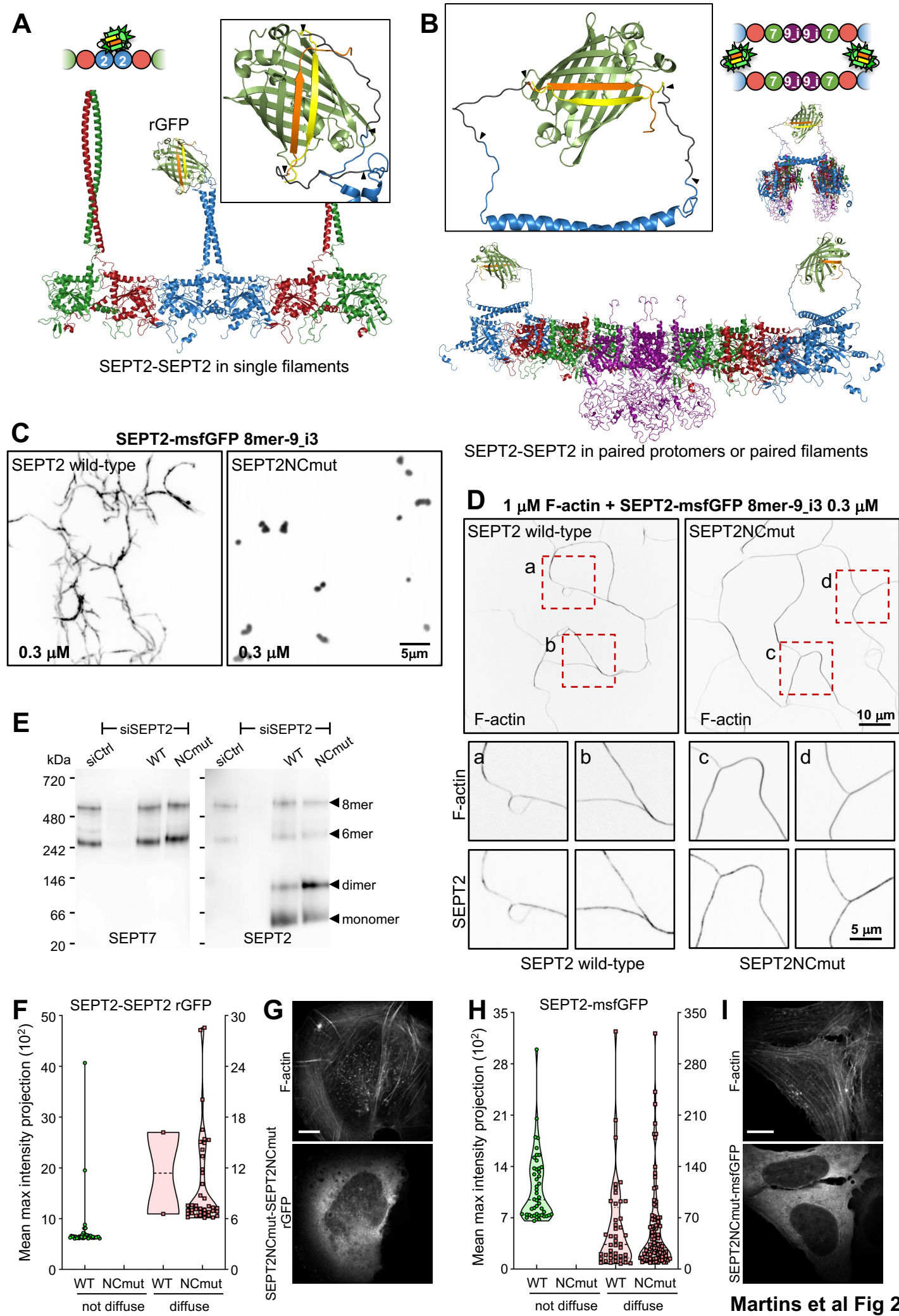
SEPT7 rGFP distribution in a live cell co-expressing mCherry-SEPT9_i1 (not shown) and labeled for microtubules (SiR-tubulin). **(I)** Representative examples of GFP1-9 cells co-expressing β 11-SEPT7Gmut2 and SEPT9_i1- β 10 with rGFP localizing to microtubules. **(J)** Representative example of a GFP1-9 cell co-expressing β 10- and β 11-SEPT7Gmut2 co-expressing mCherry-SEPT9_i1 (not shown) and labeled for microtubules (SiR-tubulin). (C-J) Scale bars in large fields of views, 10 μ m. Scale bars in insets, 2 μ m. **(K)** Representative SIM micrographs of cells (i, ii) expressing mCherry-SEPT9_i1 (not shown) co-stained for SEPT7 and α -tubulin. Scale bars in large fields of views, 10 μ m. Scale bars in insets, 2 μ m. **(L)** Box plots depict the distributions of measured widths, as the full width at half maximum (FWHM), of microtubules (MT) and MT-associated septins (SEPT7). The data points are plotted on top of the respective box plots; data points correspond to width measurements at multiple positions along MT and septin fibers and in multiple MT and septin fibers per cell in a total of 8 cells. On each box, the central mark indicates the median, and the bottom and top edges of the box indicate the 25th and 75th percentiles, respectively. The whiskers extend to the minimum and maximum values. The number of measurements is $n = 128$ and 112 for MTs and septins, respectively. The respective median values are 128 nm and 119 nm for MTs and septins, respectively.

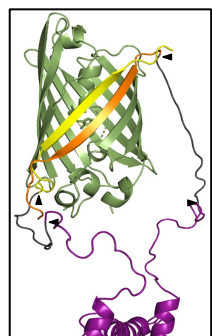
Figure S5. **Septin filaments are closely apposed to the plasma membrane, are immobilized on actin stress fibers, and can mediate actin-membrane anchoring in the absence of myosin-II.** **(A-B)** depict representative examples of lifetime decay traces for GAP43-mApple (A, left) and AF568-phalloidin (F-actin) (B, left) on glass and in the presence of gold. The solid lines represent the numerical fits, showing the lifetime reduction due to the MIET process. The respective calculated lifetime-distance dependences, used to calculate the distances of the fluorophores from the coverslip (Fig. 10E) are shown in the respective right panels. Related to Fig. 10B-E. **(C-D)** TIRF images of SEPT2-msfGFP 8mer-9_i1 alone (left) or F-actin alone (right) on top of a supported lipid bilayer (SLB) composed of 100% DOPC (C) or 5% PI(4,5)P₂ and 95% DOPC (D). Scale bar, 5 μ m. Related to Fig. 10F. **(E)** TIRF images of SEPT2-msfGFP 8mer-9_i3 and F-actin, either co-polymerized on top of an SLB, or co-polymerized in solution to form preformed bundles that were then flushed onto the supported lipid bilayer. The supported lipid bilayer was composed of 5% of PI(4,5)P₂, a septin-interacting lipid, and 95% DOPC. Scale bar, 5 μ m. Related to Fig. 10F. **(F)** TIRF images of SEPT2-msfGFP 8mer-9_i1 prepolymerized on top of a supported lipid bilayer (SLB), with actin added in a second step to the membrane-bound septin filaments, either as prepolymerized actin filaments or as G-actin. The supported lipid bilayer was composed of 5% PI(4,5)P₂ and 95% DOPC. Scale bar, 5 μ m. Related to Fig. 10F. **(G-H)** Box plots displaying the median (notch) and mean (square) \pm percentile (25–75%) of diffusion coefficients corresponding to the free diffusion (D_{diff}) (G) and confined diffusion (D_{conf}) (H) trajectories outside FAs from sptPALM. Related to Fig. 10G-K. Statistical significance was obtained using two-tailed, non-parametric Mann–Whitney rank sum test. The different conditions were compared to

the SEPT9_j3-mEos3.2 condition. The resulting P values are indicated as follows: ** P<0.01; **** P < 0.0001. **(I)** Representative confocal micrographs of U2OS cells co-stained for F-actin (phalloidin), SEPT7 and non-muscle myosin heavy chain isoforms IIA (NMIIA) or IIB (NMIIB). Wild-type U2OS cells were co-stained for either NMIIA (i) or NMIIB (ii). NMIIA knock-out (KO) U2OS cells were co-stained for NMIIB (iii). NMIIB KO U2OS cells were co-stained for NMIIA (iv). **(J)** Western blot of NMIIA KO and NMIIB KO U2OS cell lysates probed with anti- α -tubulin, anti-SEPT7, anti-NMIIA and anti-NMIIB antibodies. NMIIA KO cells were treated either with LacZ or NMIIB siRNA. NMIIB KO cells were treated either with LacZ or NMIIA siRNA. Molecular weight markers are shown on the left. The condition shown with the red rectangle corresponds to the images shown in panel (K). **(K)** Representative confocal micrographs of NMIIA KO U2OS cells (i-iii) treated with NMIIB siRNA and co-stained for F-actin (phalloidin), SEPT7 and NMIIB. Scale bars in large fields of views, 10 μ m. Scale bars in insets, 2 μ m.

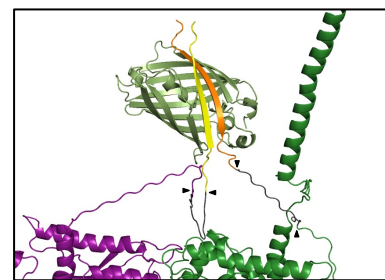
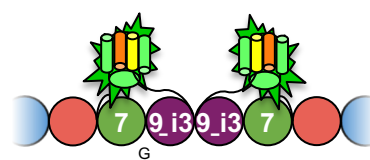


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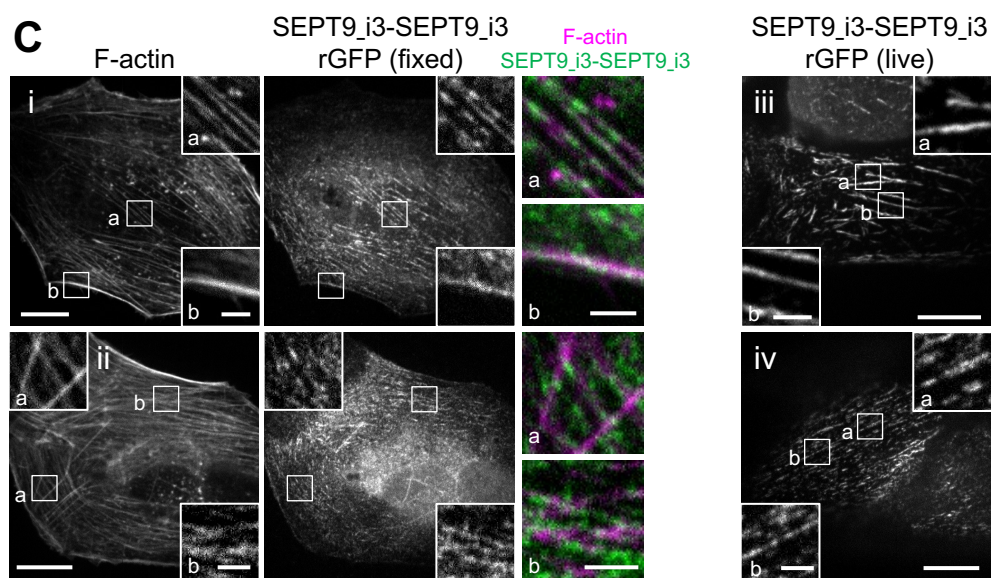
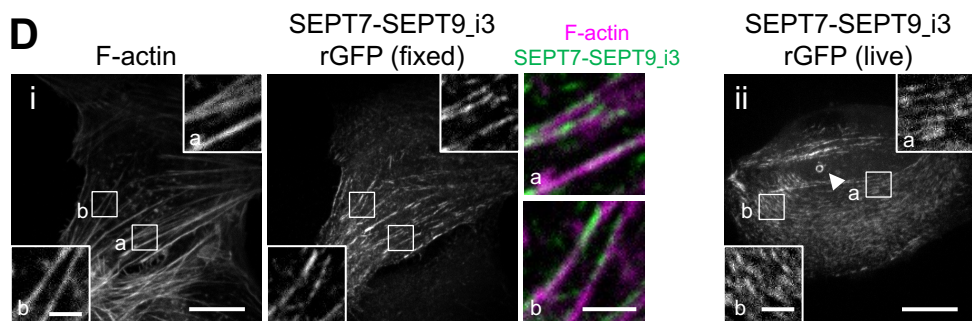
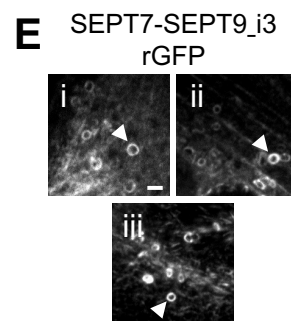
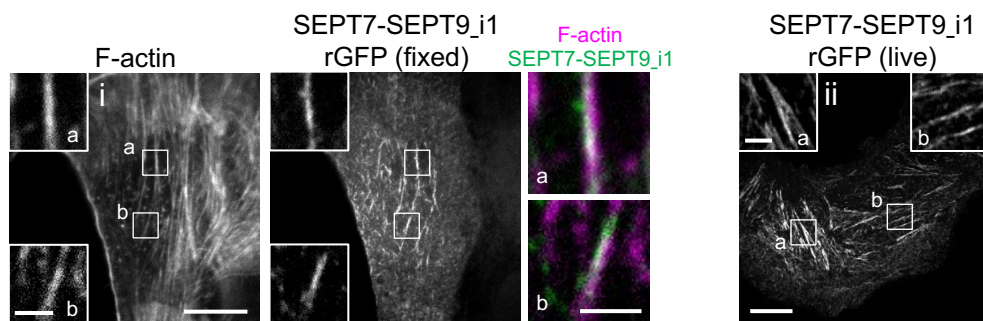


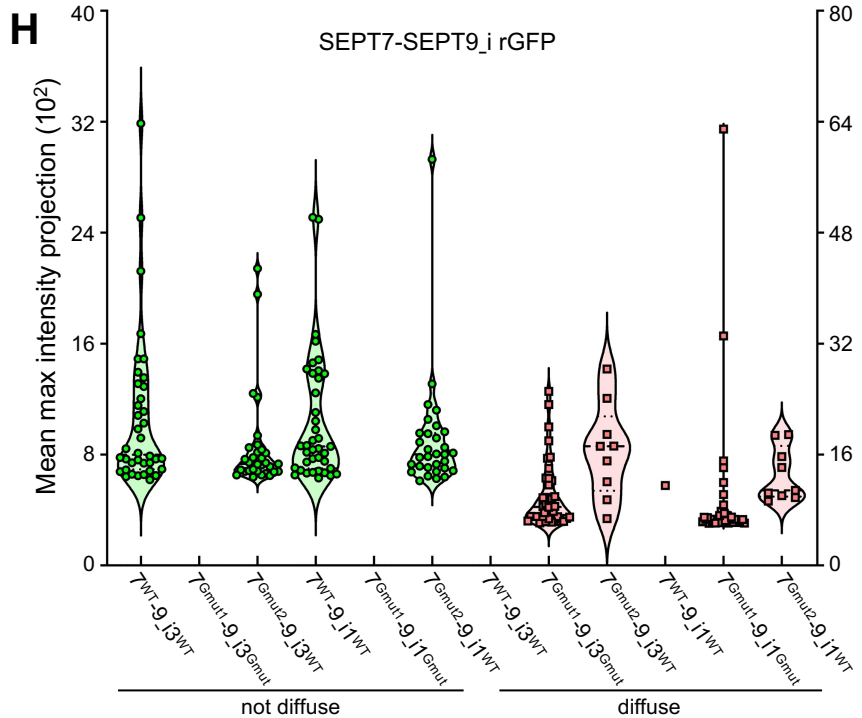
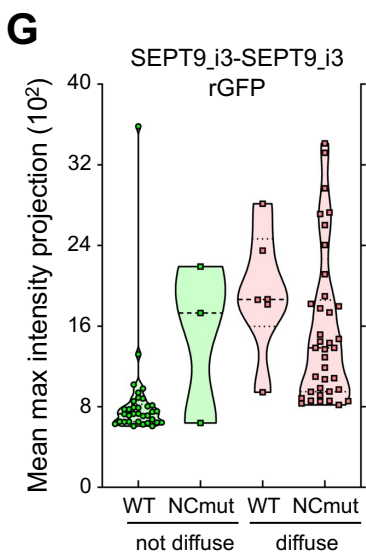
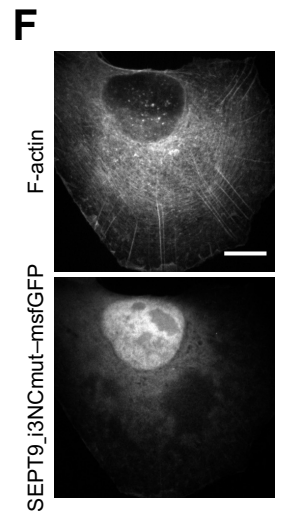
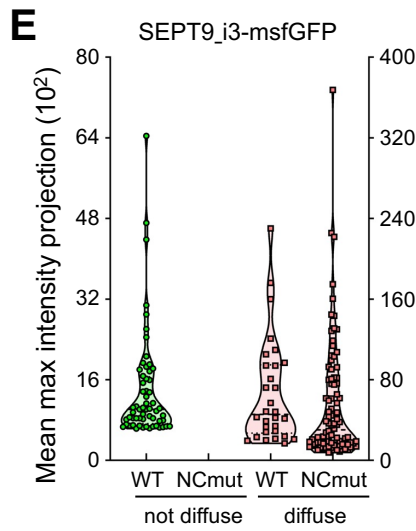
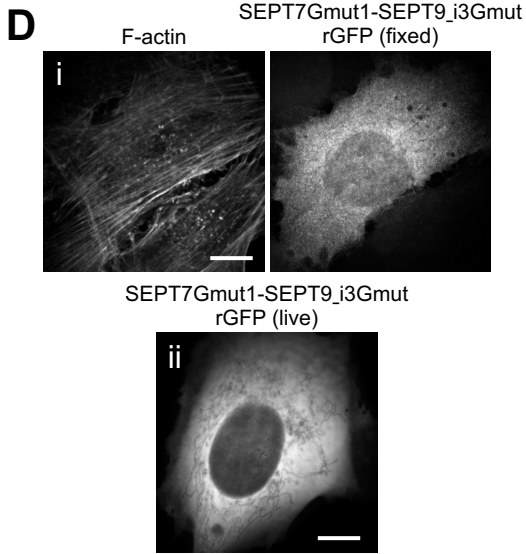
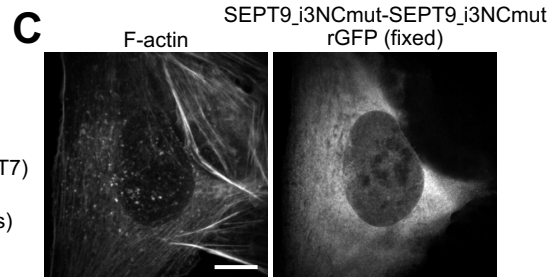
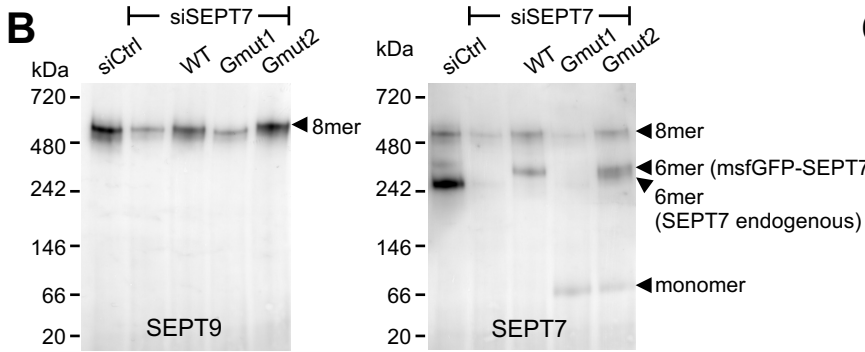
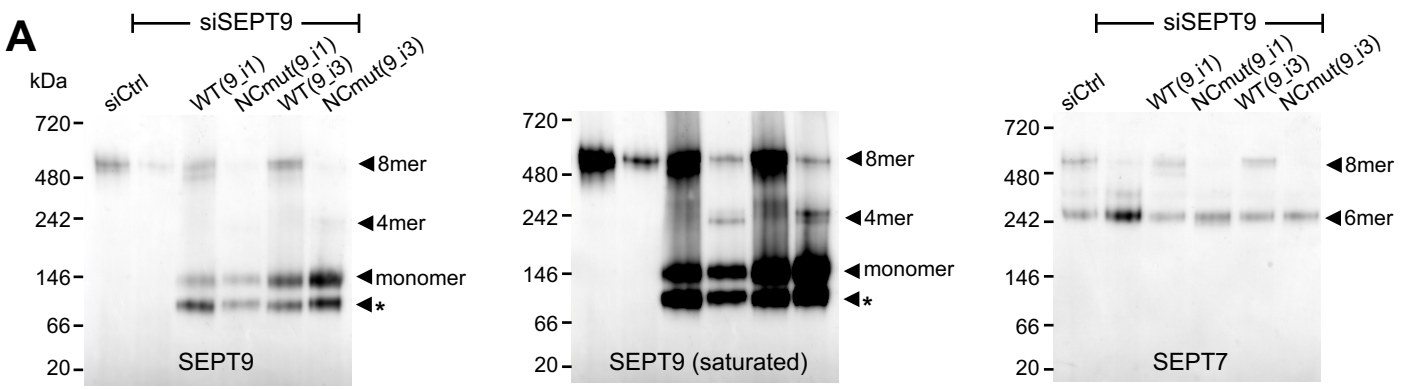
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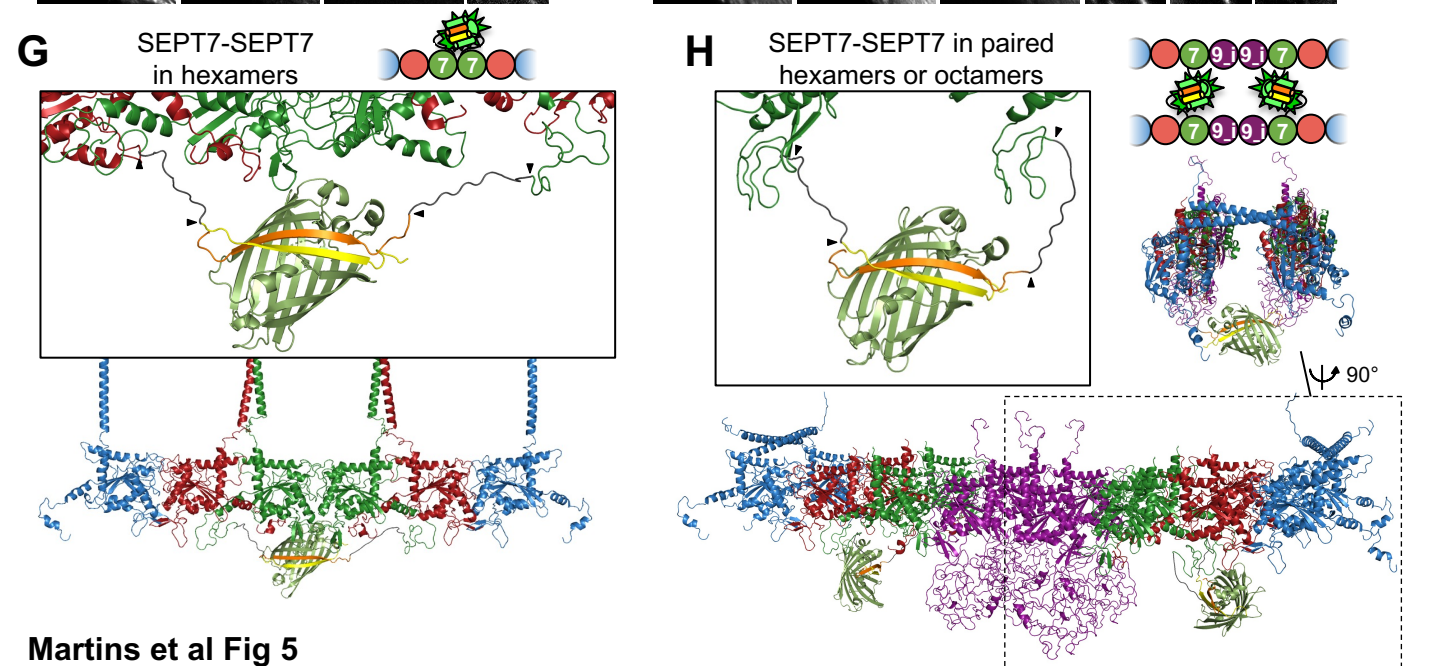
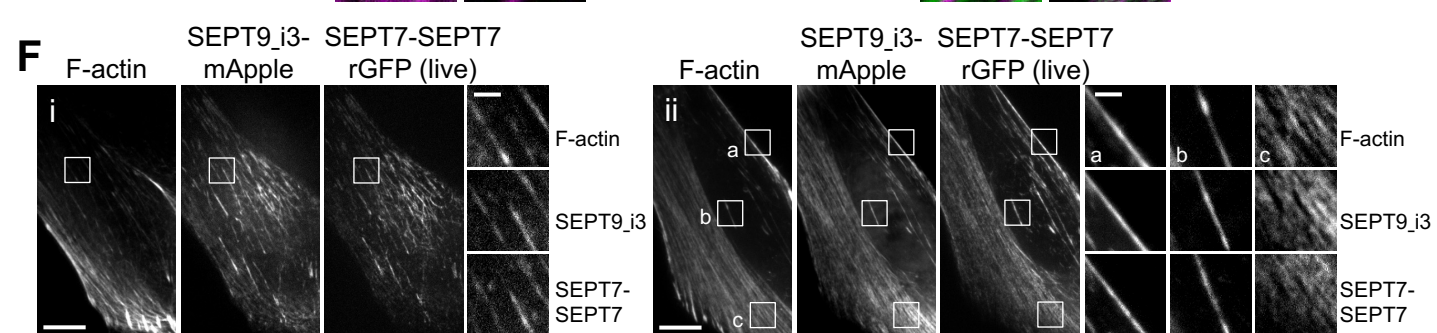
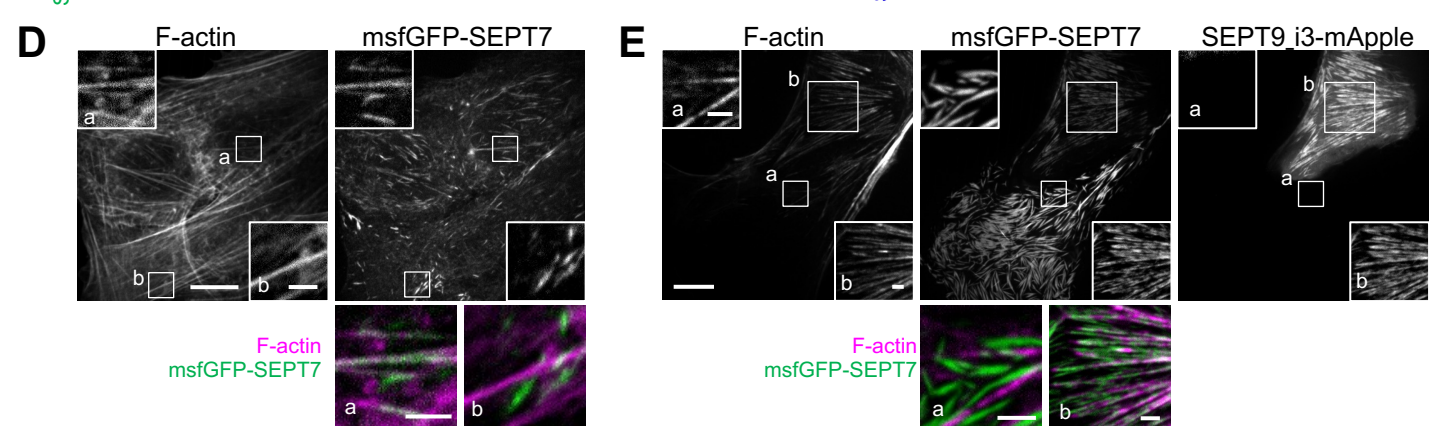
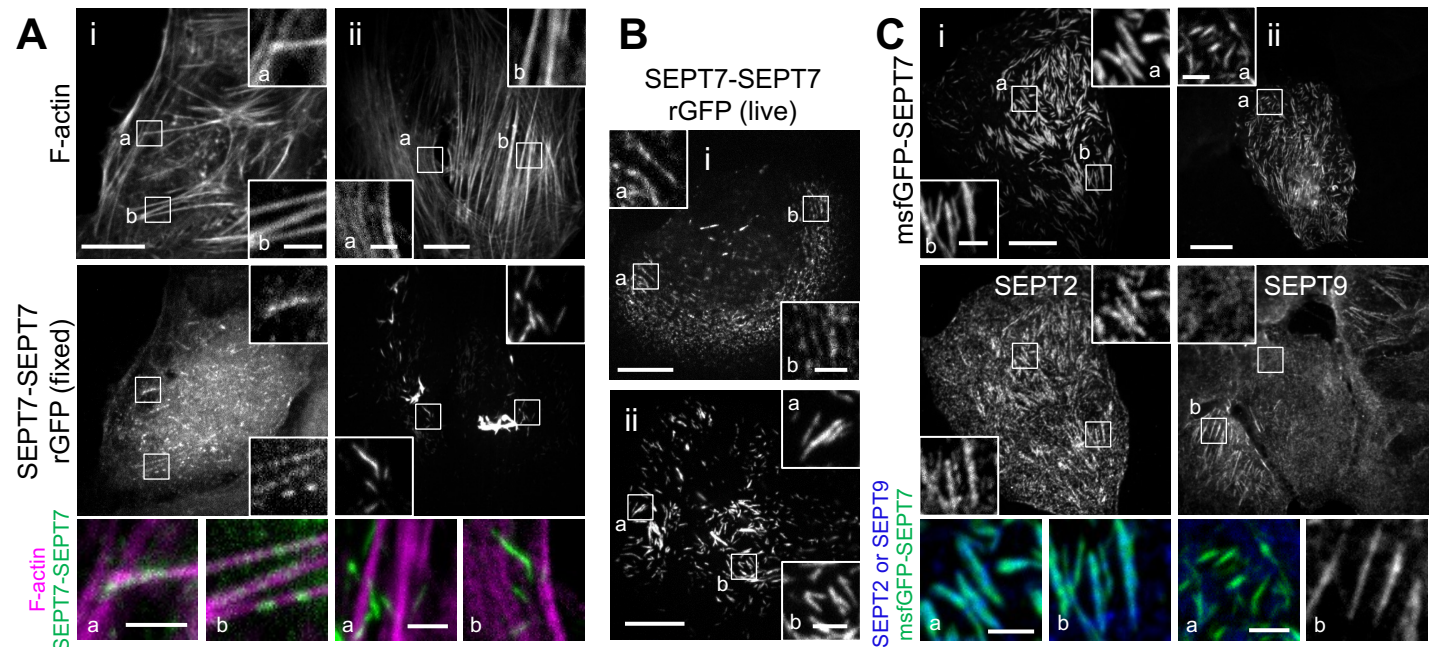
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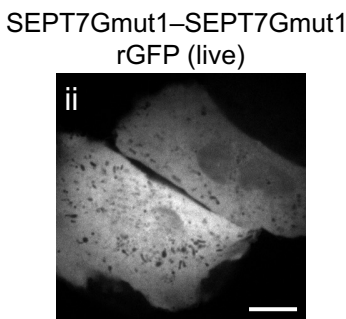
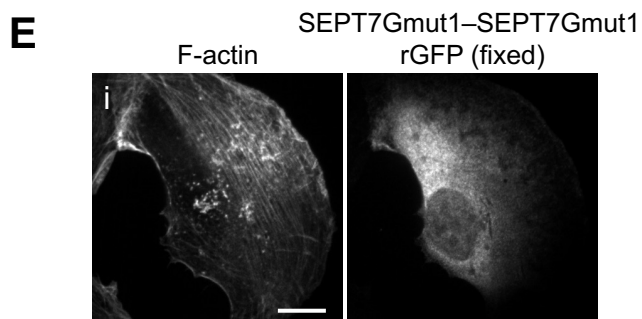
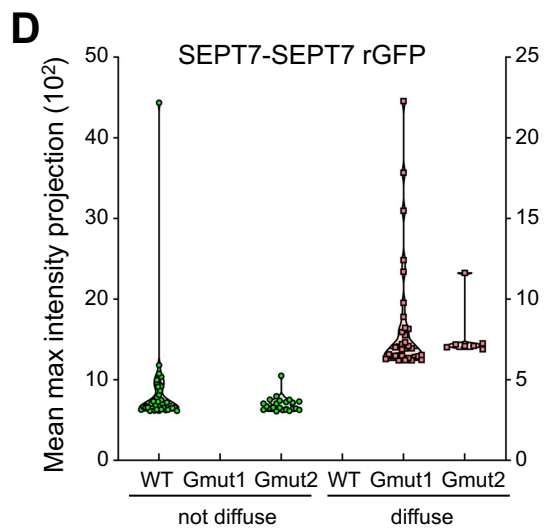
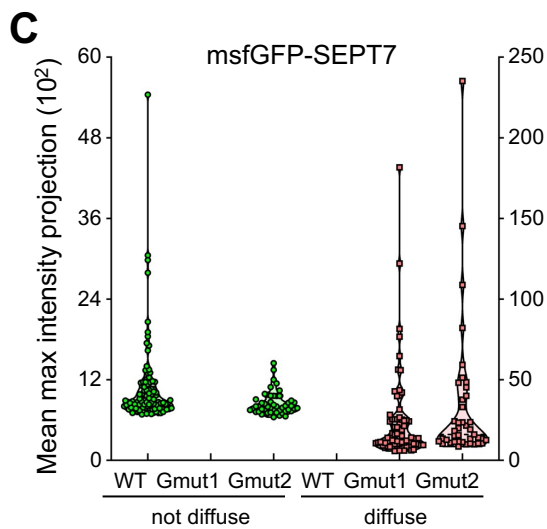
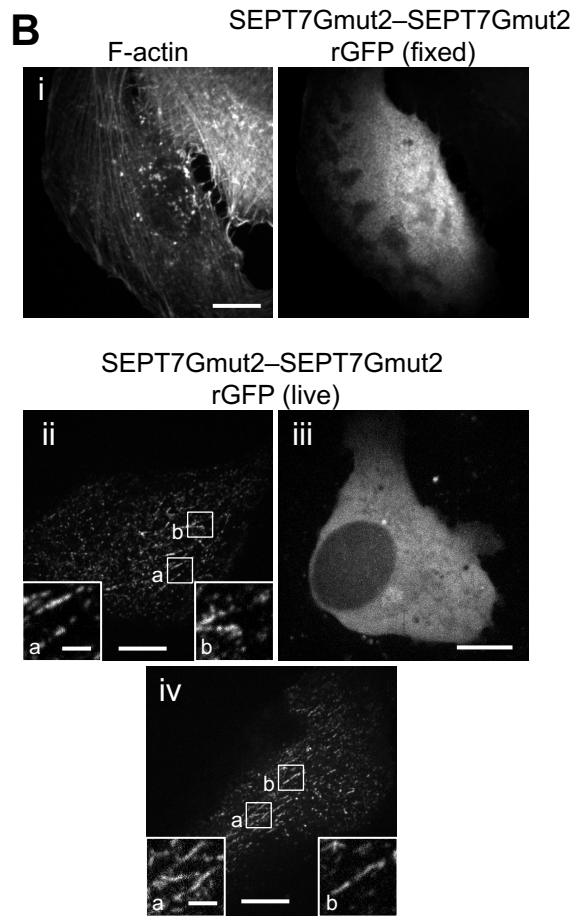
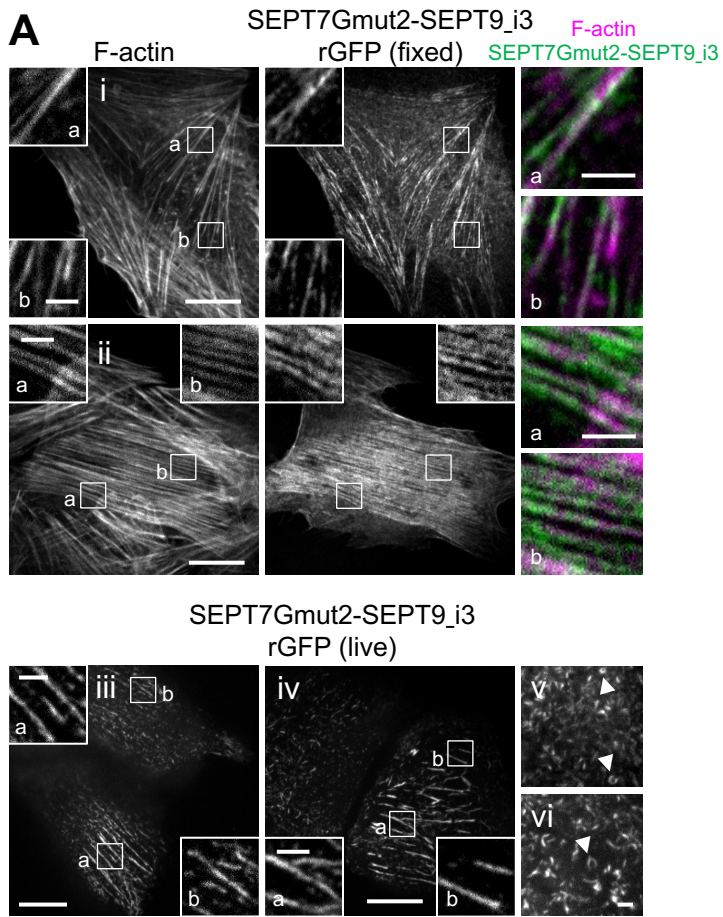
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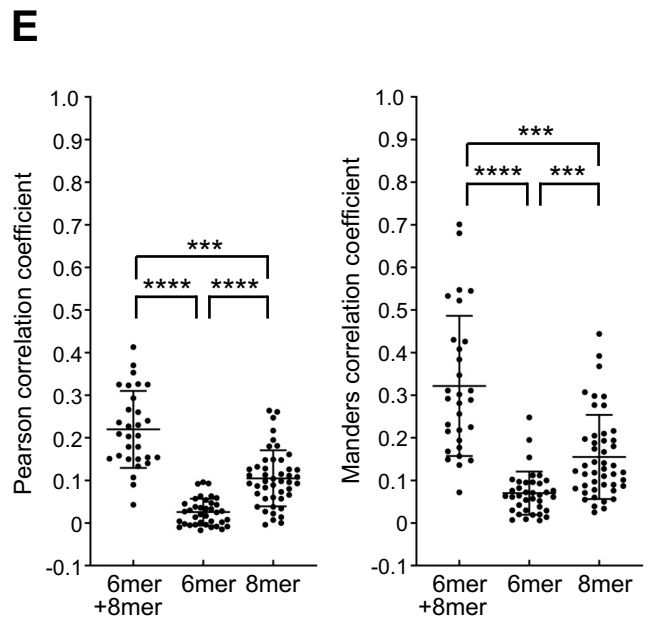
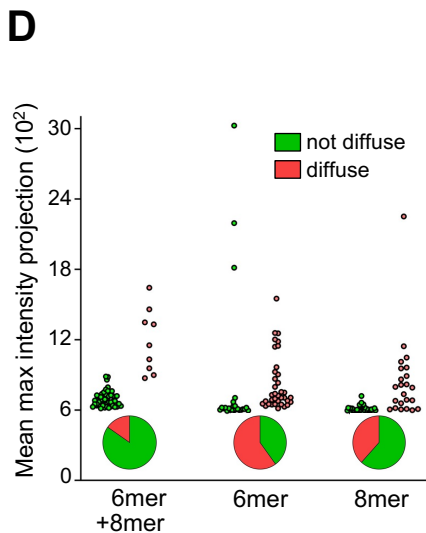
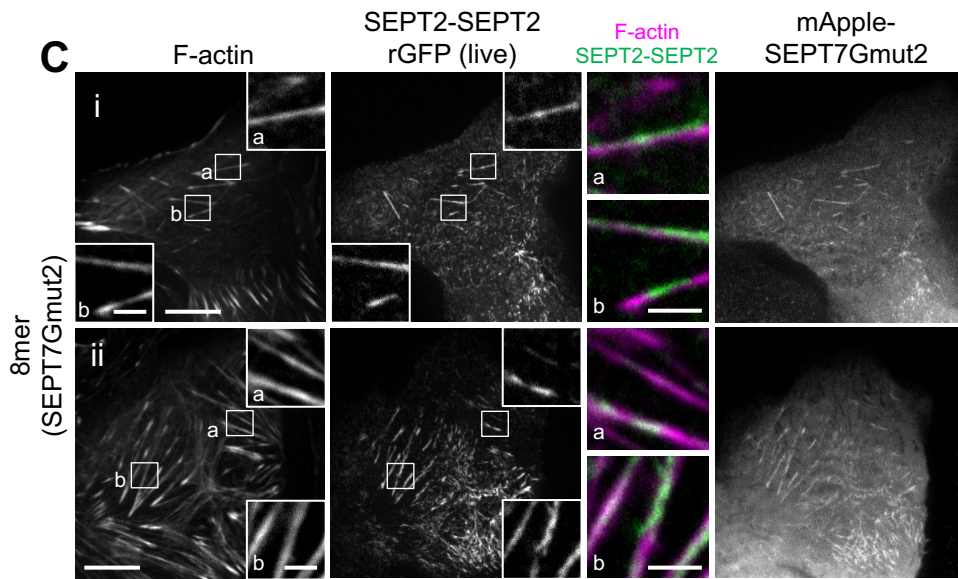
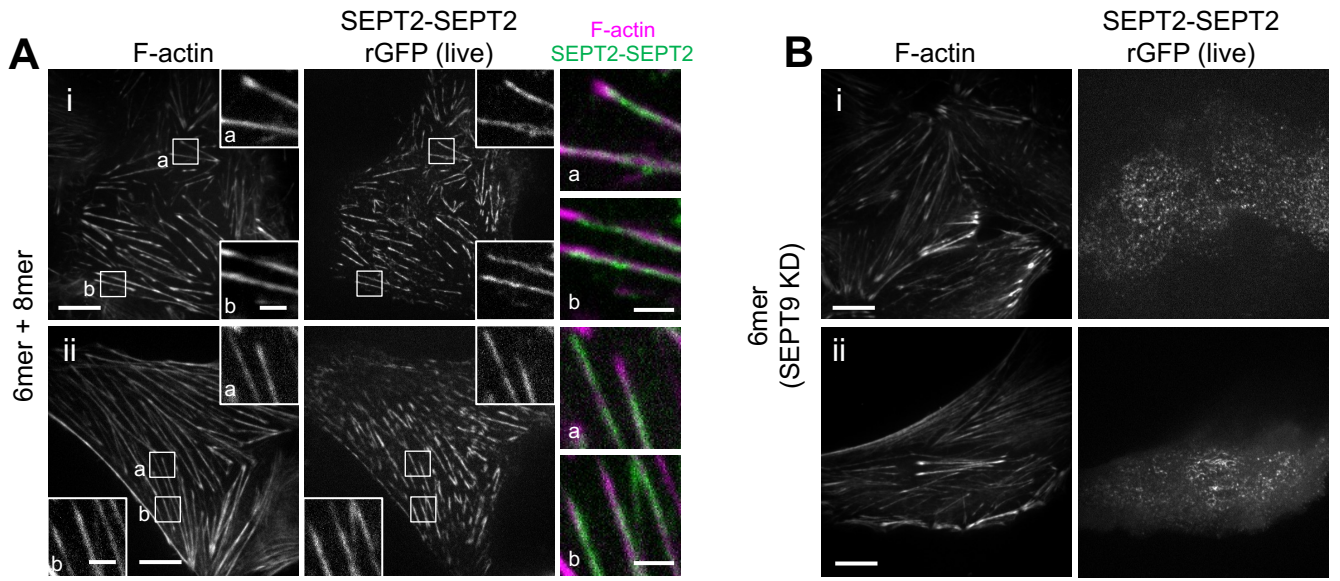
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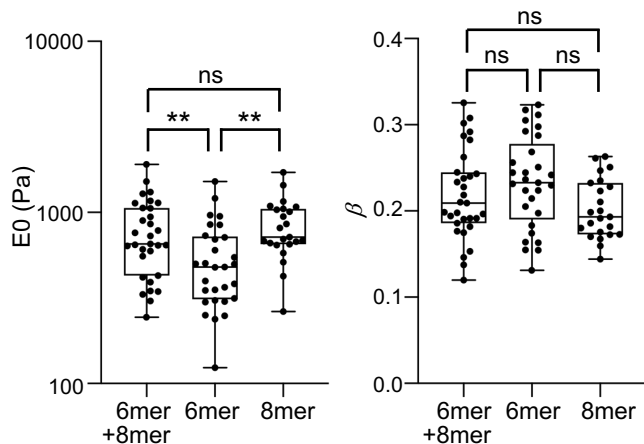
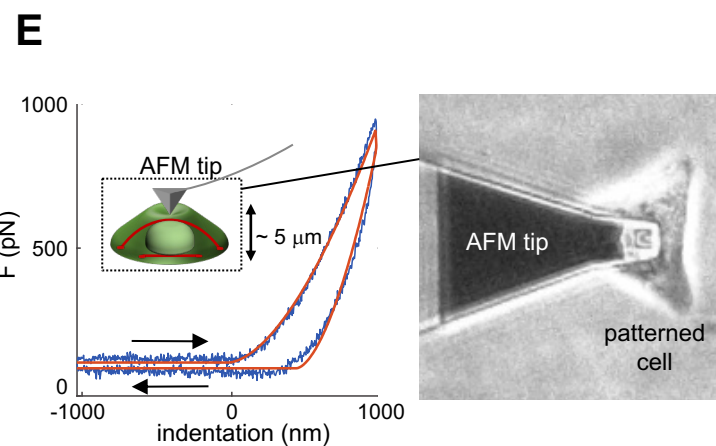
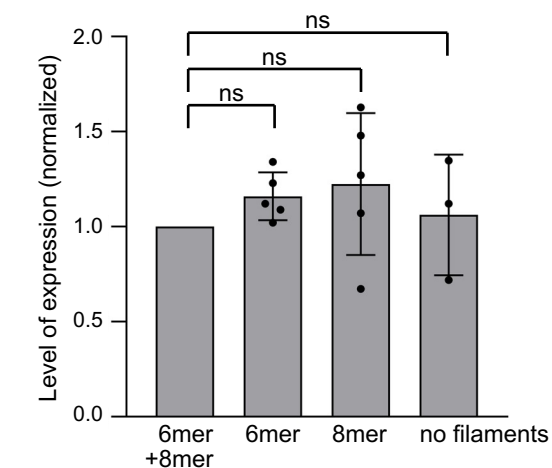
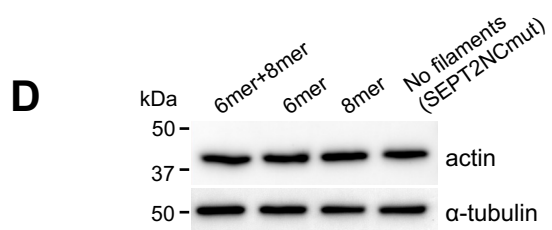
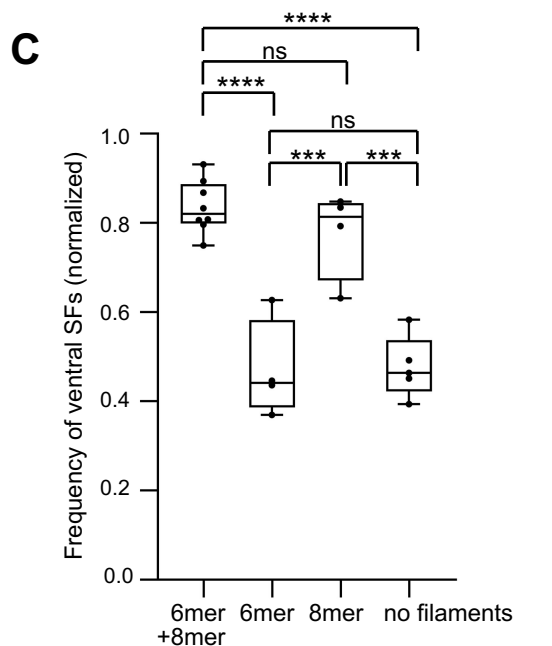
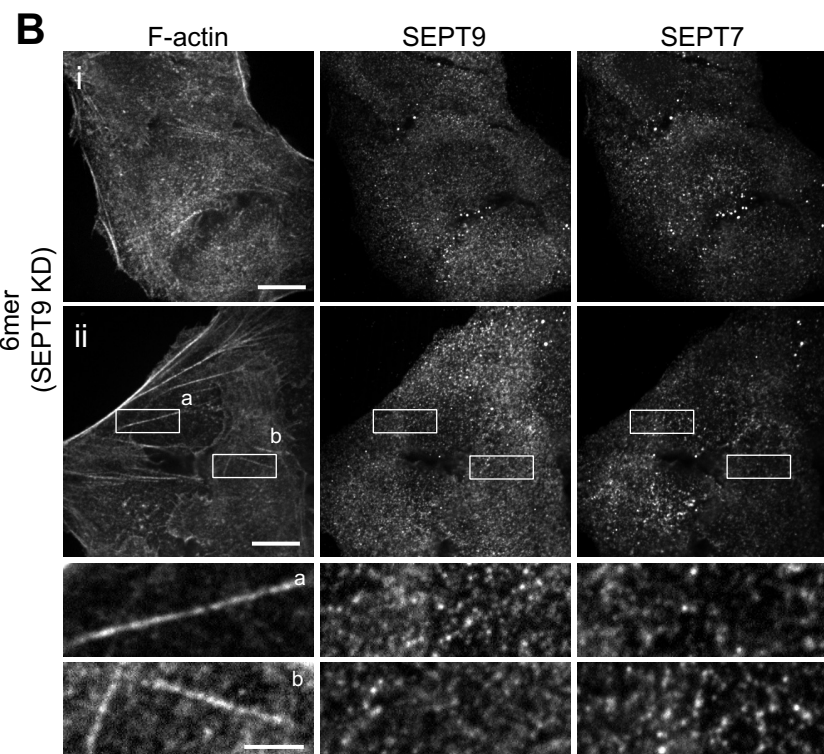
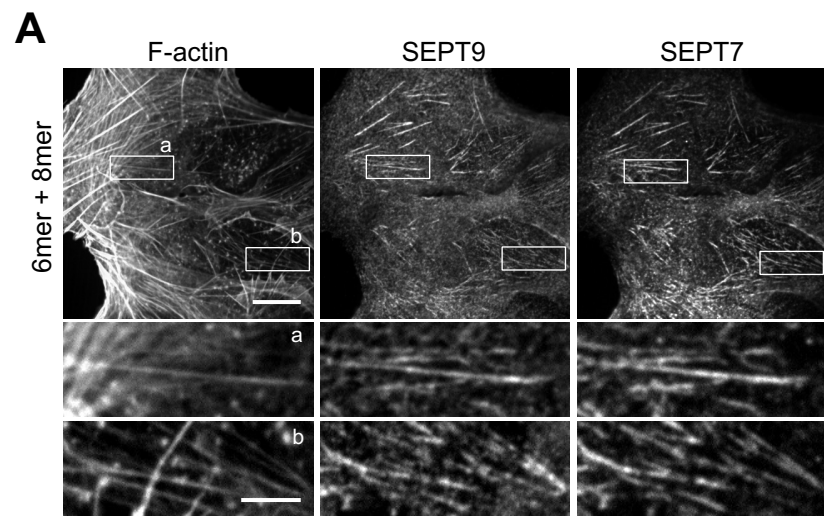
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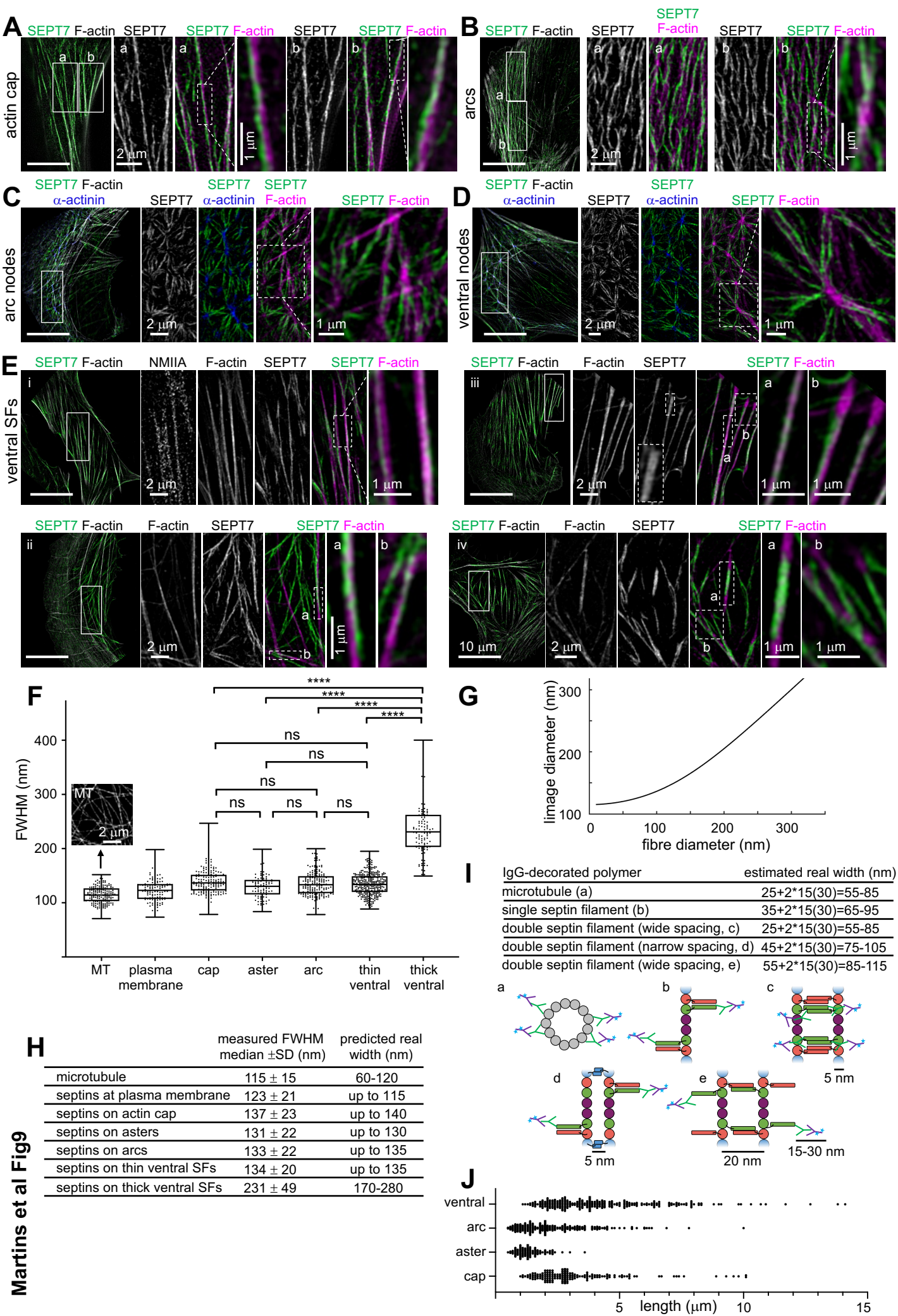


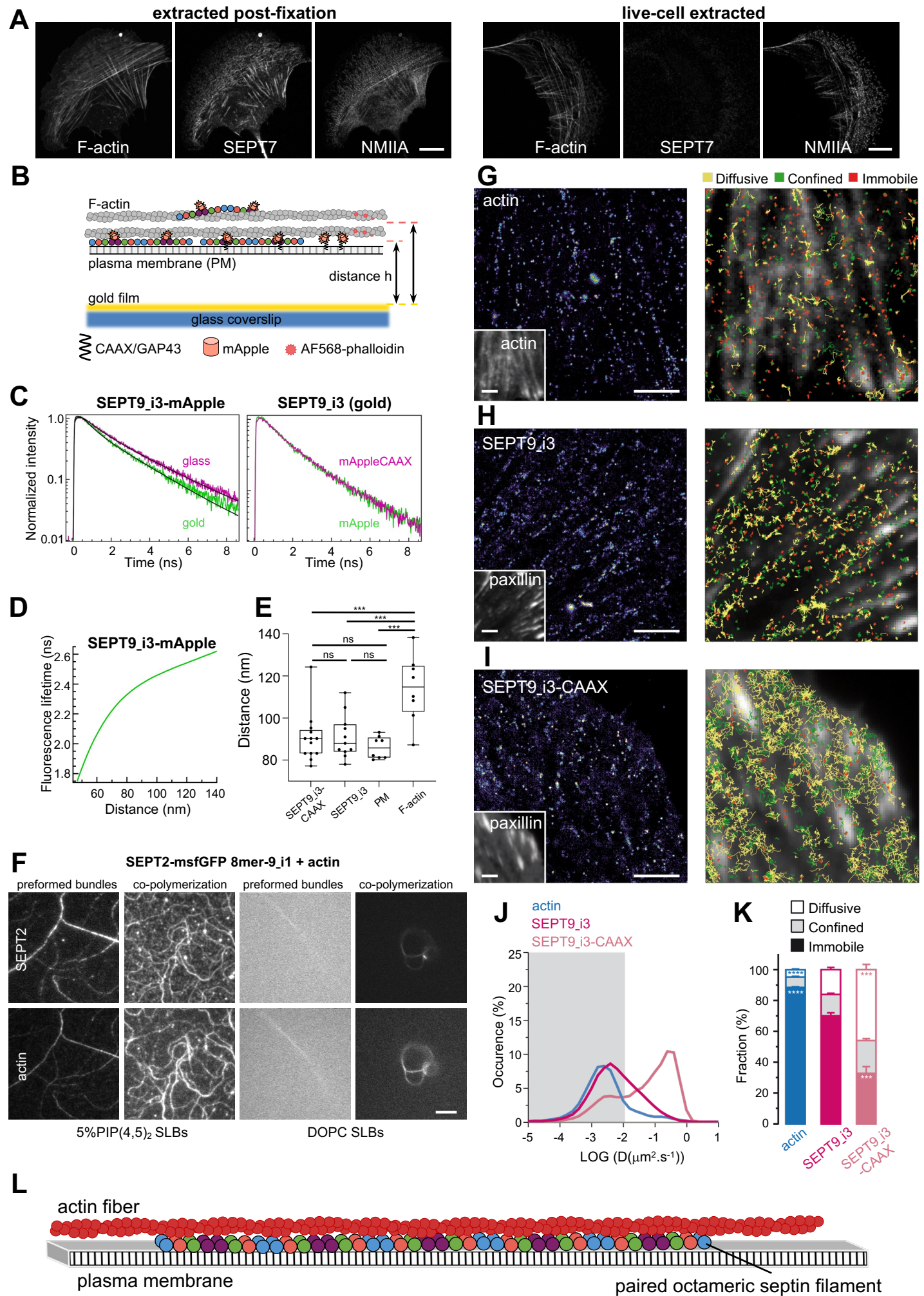


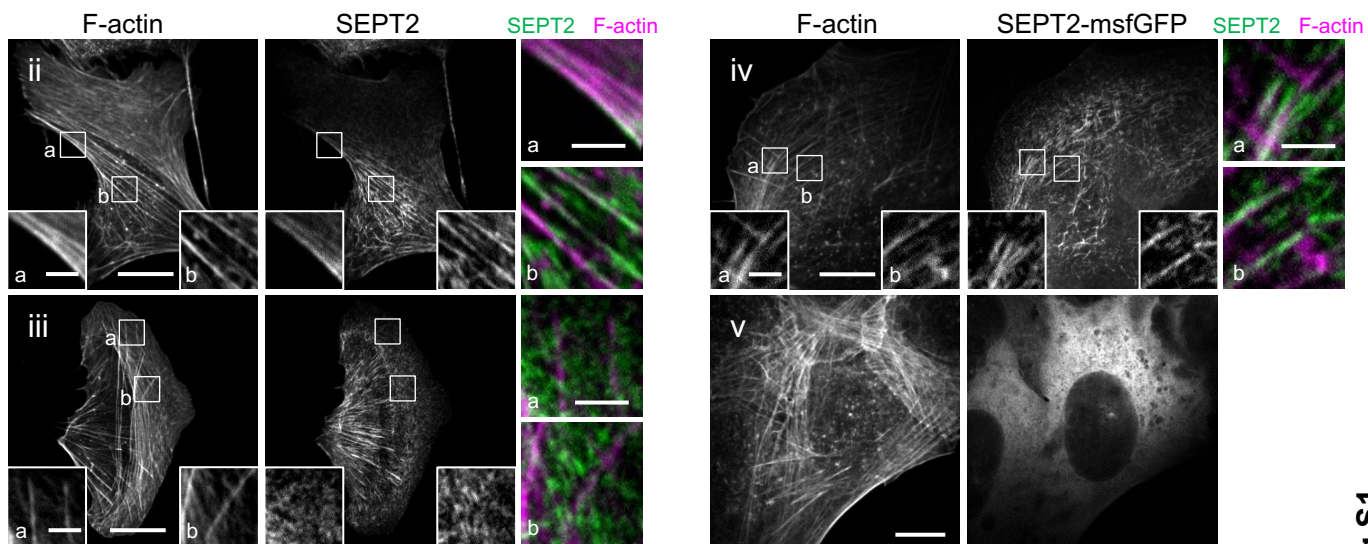
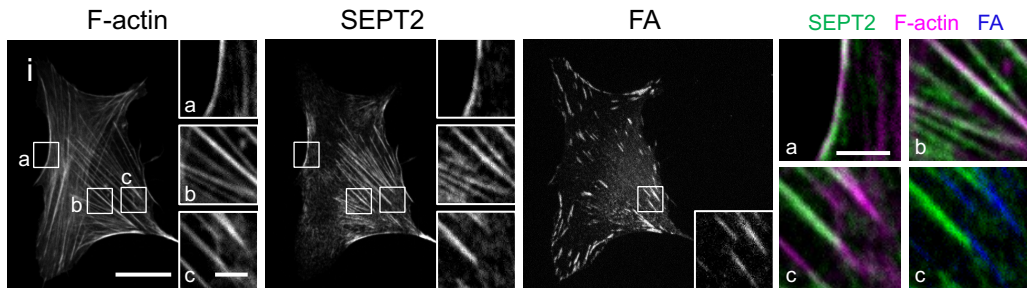
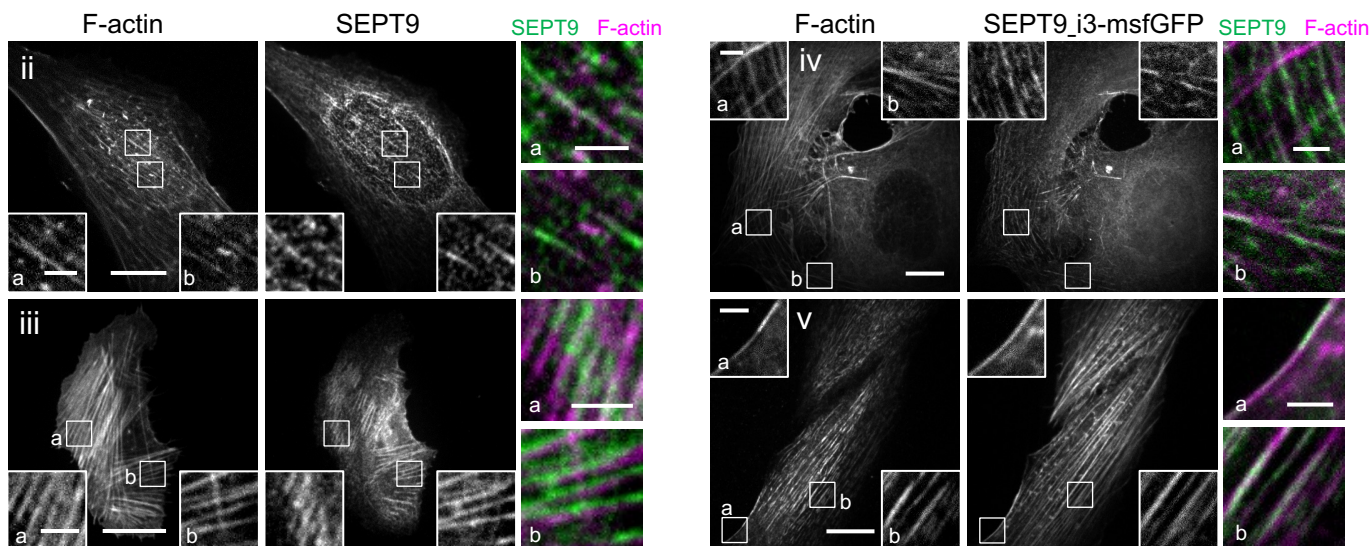
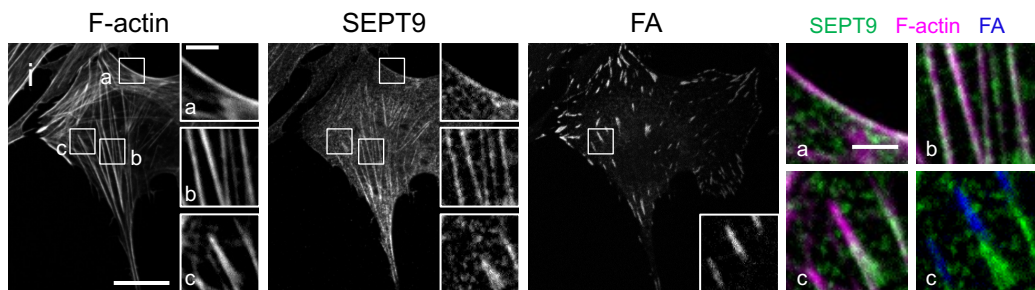
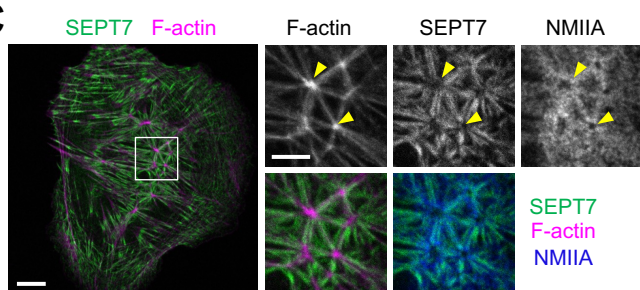
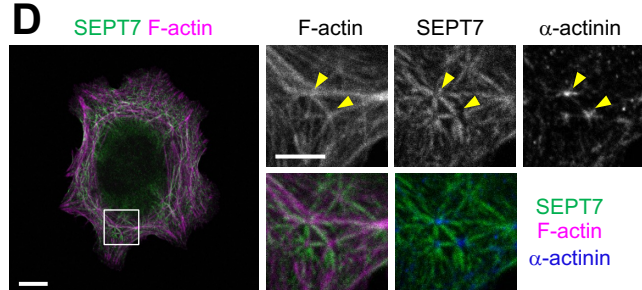




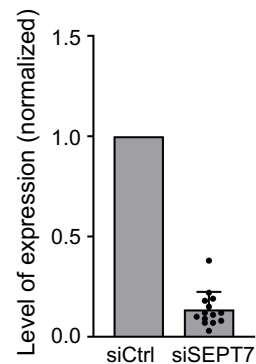
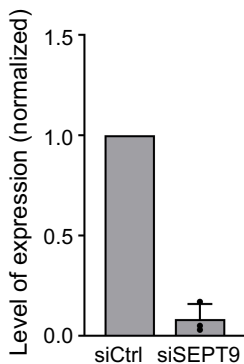
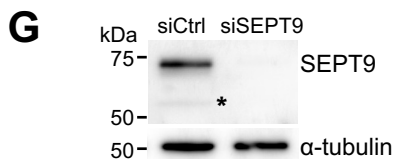
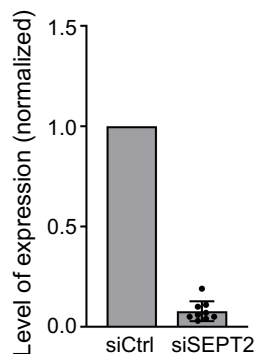
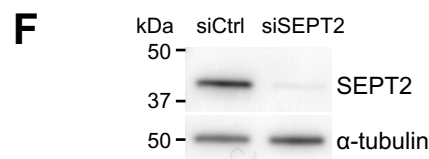
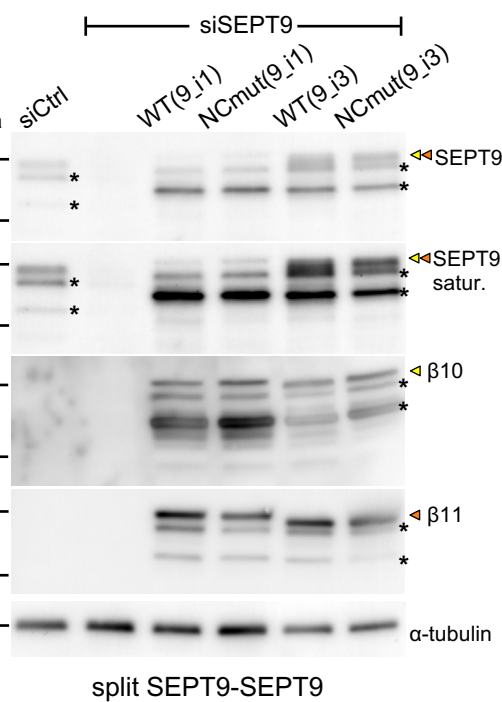
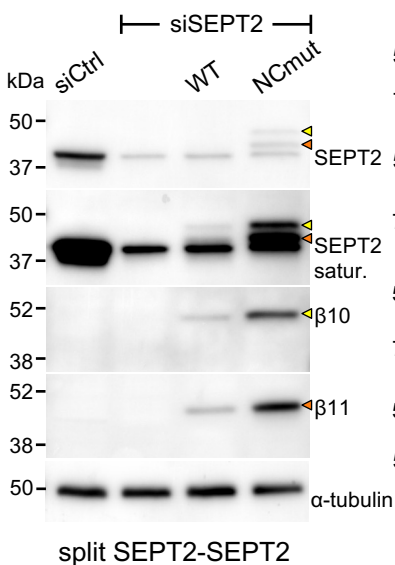
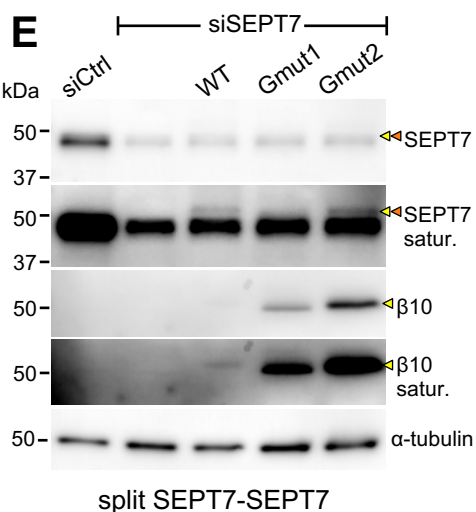
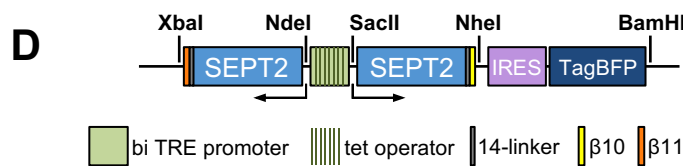
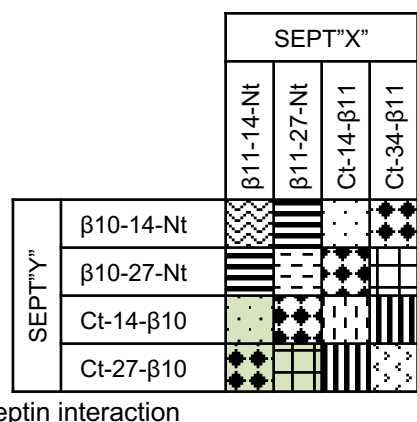
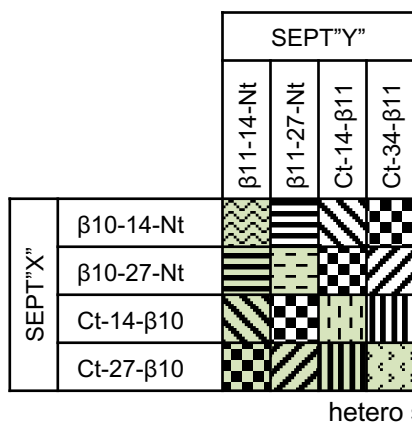
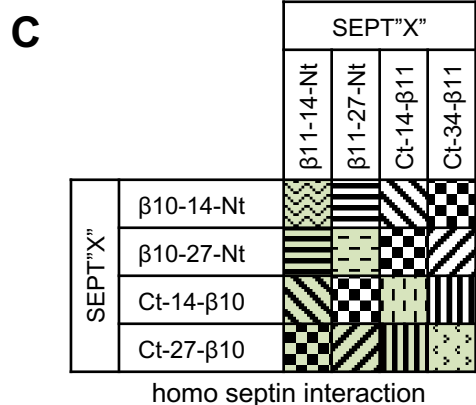
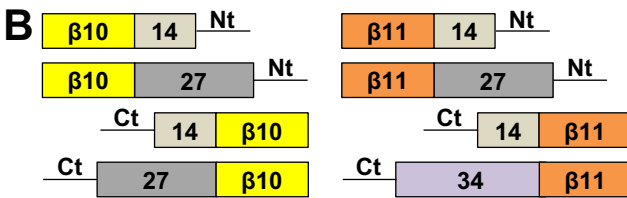


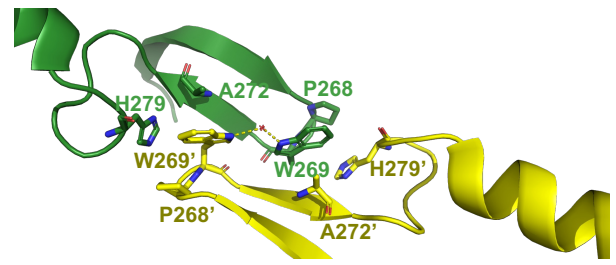
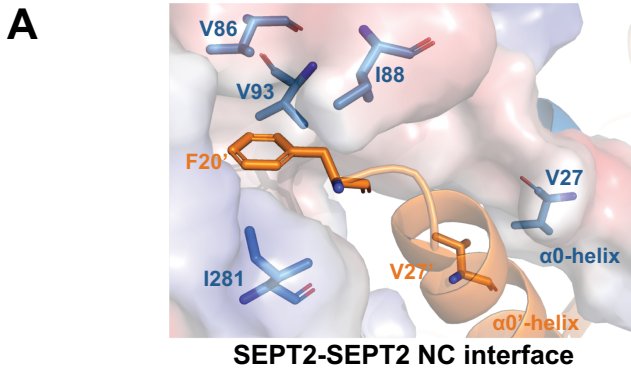




A**B****C****D**

A 34-linker IDGAGGSPGGGSGGSGSGGGGSGGGGSASGGSTS
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 β 10 MGDLPDDHYLSTQTILSKDLN
 β 11 EKRDHMLVLEYVTAAGITDAS





	α 0-helix		β 2		α 5
SEPT2	20 27		86 88 93		281
	<u>G</u> FANLPNO <u>V</u> HRKSVKKG... <u>E</u> ASTV <u>E</u> I <u>E</u> ERG <u>V</u> KLR... <u>L</u> RTML <u>I</u>				
SEPT9_i3	263 270		329 331 336		523
	<u>G</u> IDSILE <u>Q</u> MRRKAMKQG... <u>K</u> SIT <u>H</u> D <u>I</u> E <u>E</u> K <u>G</u> VRMK... <u>L</u> RDLL <u>I</u>				
	*: : *: : *		::* .*: :		** :::

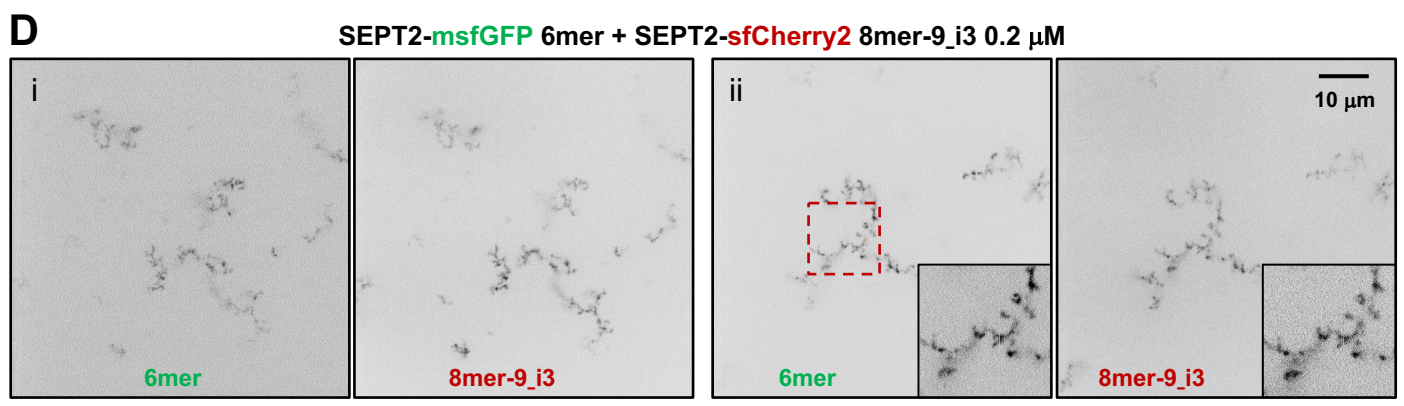
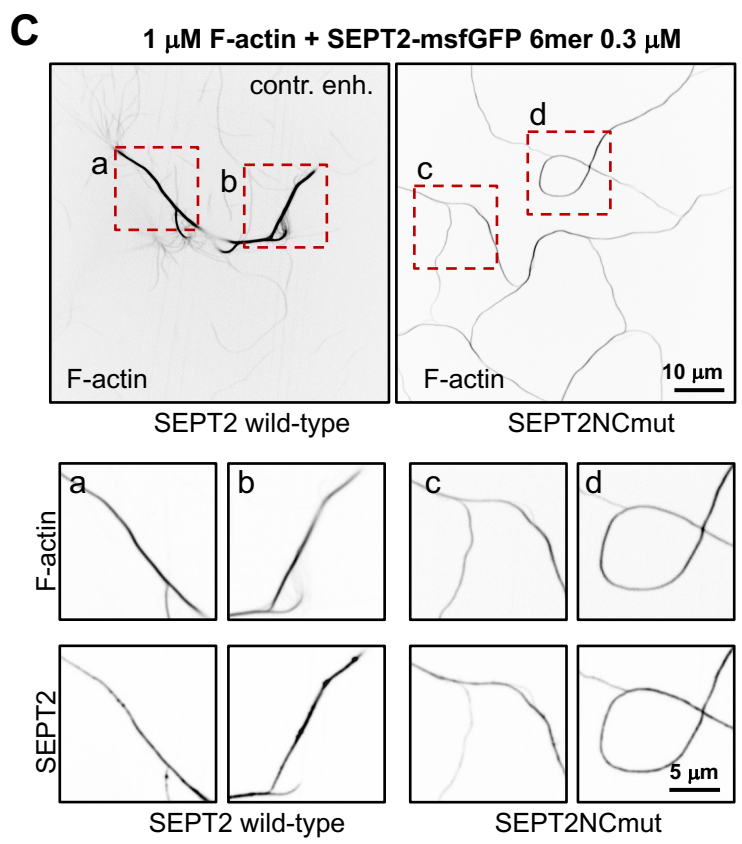
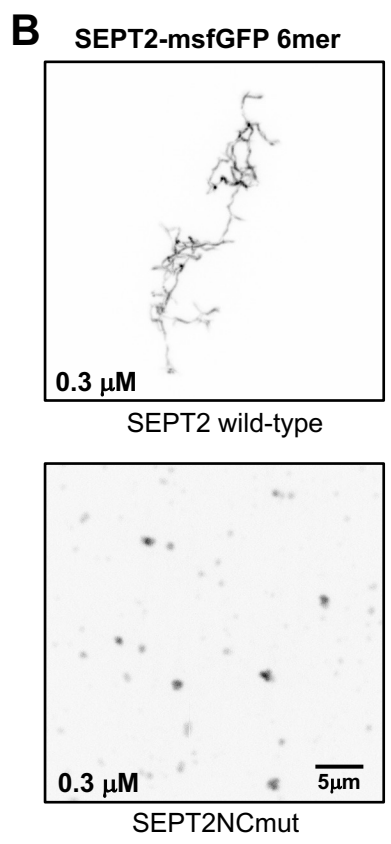
	β 7	β 8
SEPT7	269 272 279	
	<u>Q</u> Y <u>P</u> W <u>G</u> V <u>A</u> EVNGE <u>H</u>	
SEPT9_i3	502 505 512	
	<u>K</u> T <u>K</u> W <u>G</u> T <u>I</u> EVENT <u>T</u> H	
	** *: *	

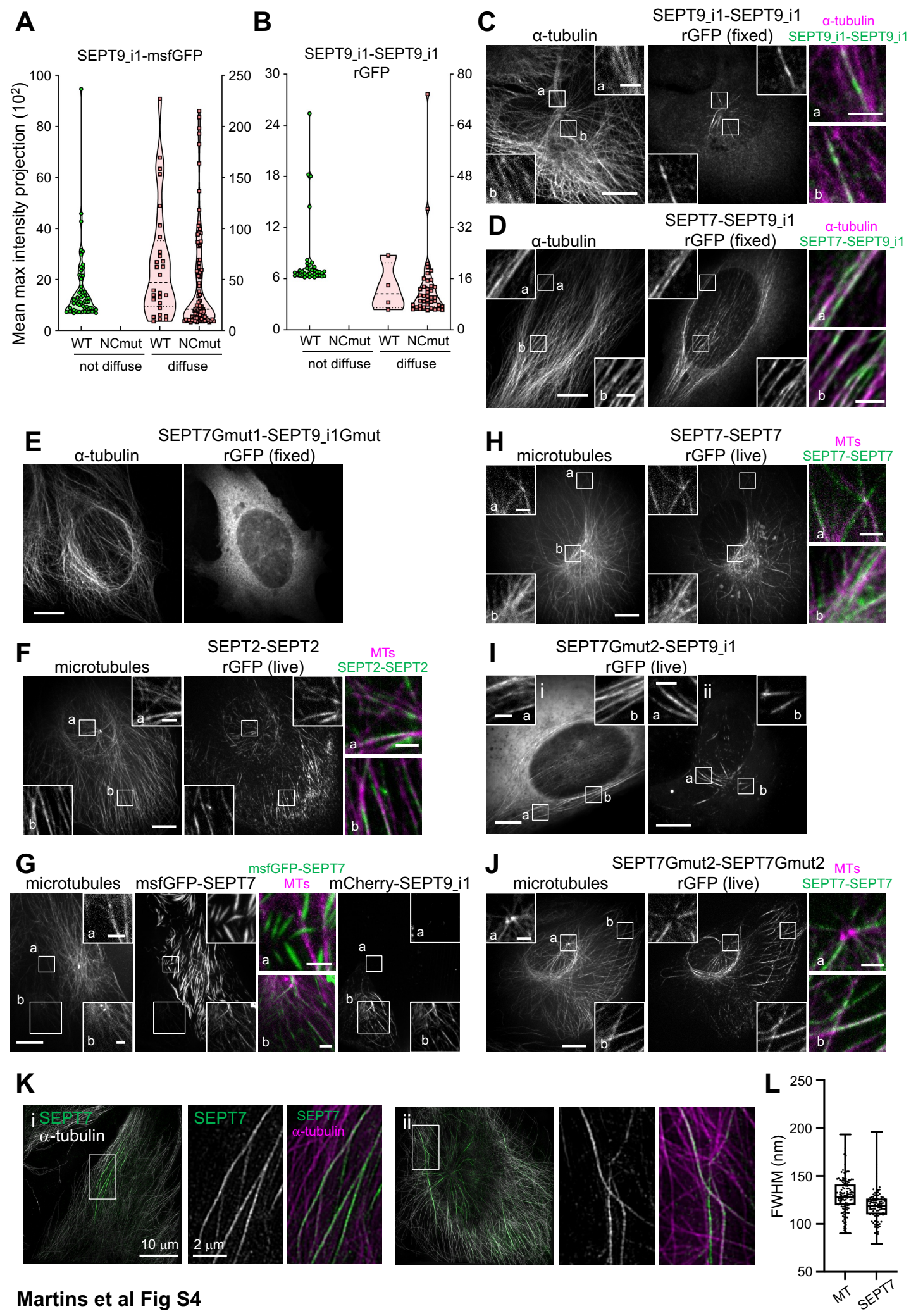
NC interface mutants

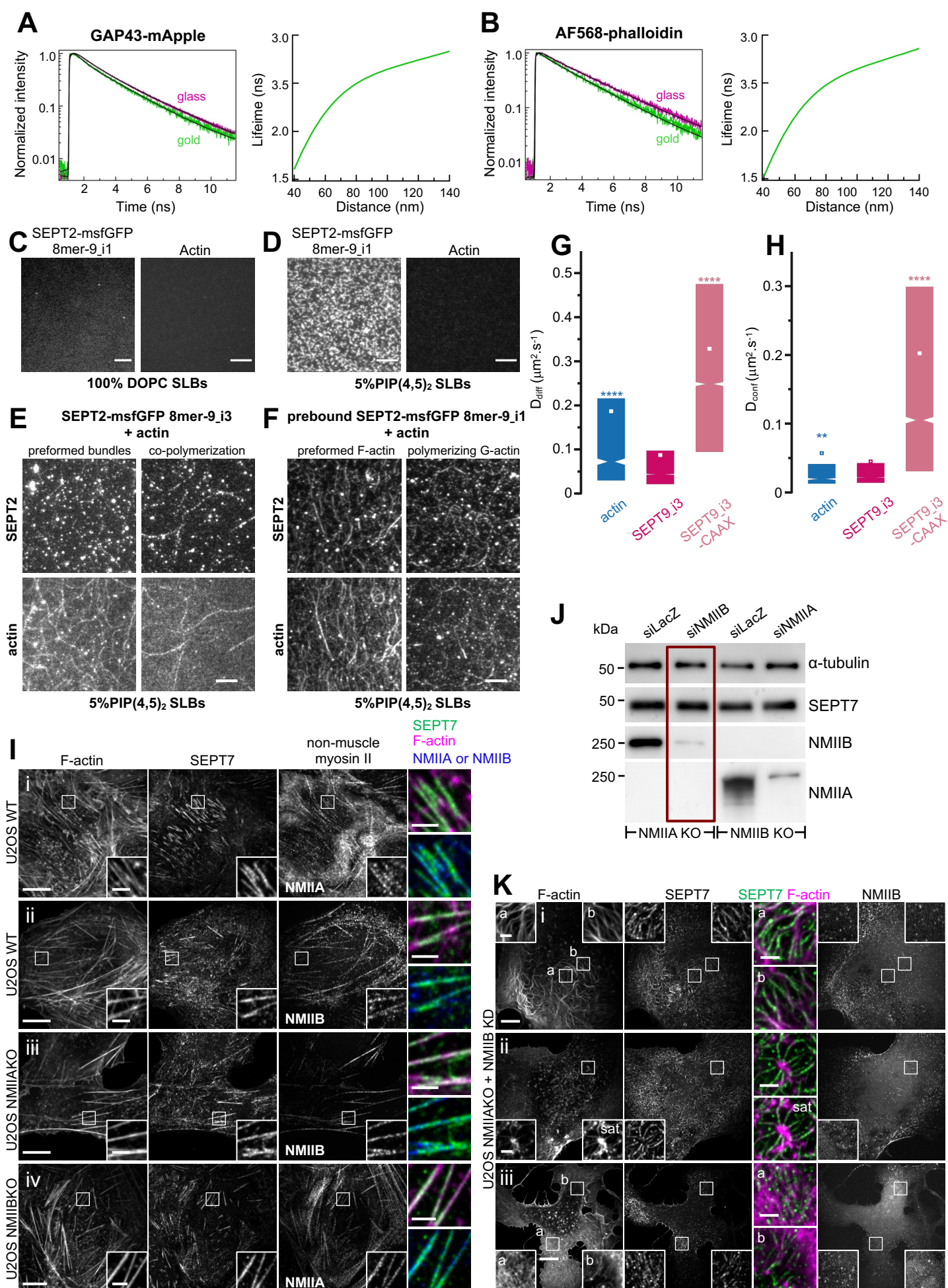
SEPT2NCmut	F20D, V27D
SEPT9_i1NCmut	I281D, M288D
SEPT9_i3NCmut	I263D, M270D

G interface mutants

SEPT7Gmut1	W269A, H279D
SEPT7Gmut2	H279D
SEPT9_i1Gmut	W520A, H530D
SEPT9_i3Gmut	W502A, H512D







Full length FP fusions in pCMV		
Construct name	Primer name	Primer sequence
SEPT2-msfGFP	NheIhSept2FOR	CGTCAGATCCGCTAGCATGTCTAAGCAACAACCAACTCAGT
	hSept2sfGFPFOR	CCATGTGGTGTCCAAGGGCGAGGAGC
	hSept2sfGFPREV	TTGGACACCACATGGTGGCCGAGAGC
msfGFP-SEPT7	BamHIsfGFPREV	TAGATCCGGTGGATCCTTACTTGTACAGCTCATCCATGCC
	NhelmsfGFPFOR	CGTCAGATCCGCTAGCATGGTGTCCAAGGGCGAGG
	msfGFPSept7i1FOR	GTCAAGTCGGTCAGTCCGAGATCCG
	msfGFPSept7i1REV	CTGACCGACTTGTACAGCTCATCCATGCCAG
SEPT9_i3-mApple	BamHISept7i1REV	TAGATCCGGTGGATCCTTAAAAGATCTTCCCTTTCTTCTTGTTCCTTTCCAAGG
	NheISept9_i3FOR	CGTCAGATCCGCTAGCATGGAGAGGGACCGGATCTC
	mAppleFOR	GTGAGCAAGGGCGAGGAG
	mAppleSep9REV	CTCGCCCTTGCTCACCATCTCTGGGGCTTCTGGC
GAP43-mApple	BamHImAppleREV	TAGATCCGGTGGATCCTCACTTGTACAGCTCGTCCATGCC
	NheIGAP43mAppleFOR	CGTCAGATCCGCTAGCATGCTGTGCTGTATGCCAAGAACCAAACAGTTGAAAAAATGATGAGGACCAAAAGATTGTGAGCAAGGGCGAGGAG
	BamHImAppleREV	TAGATCCGGTGGATCCTCACTTGTACAGCTCGTCCATGCC
SEPT9_i3-mApple-CAAX	NheISept9_i3FOR	CGTCAGATCCGCTAGCATGGAGAGGGACCGGATCTC
	mAppleFOR	GTGAGCAAGGGCGAGGAG
	mAppleSep9REV	CTCGCCCTTGCTCACCATCTCTGGGGCTTCTGGC
	BamHICAAXsfGFPREV	TAGATCCGGTGGATCCTTAGGAGAGCACACACTTGCAGCTCATGCAGCCGGGGCCACTCTCATCAGGAGGGTTCAGCTTCTTGTACAGCTCATCCATGCC
SEPT2NCmut-msfGFP	NdeICMVpromoterFOR	ATCAAGTGATCATATGCCAAGTAGCAGCCCTATTGACGTCAATGACG
	Sept2FDFOR	CAAATCTTCCCAATCAGATCACCAGAAATCAGTGAAGAAGGGTTCCG
	Sept2FDREV	GATTGGGAAGATTTGCATCTCCAACATAGCCAGGAGTTTCTGG
	BamHIsfGFPREV	TAGATCCGGTGGATCCTTACTTGTACAGCTCATCCATGCC
SEPT9_i3NCmut-msfGFP	NheISept9_i3FOR	CGTCAGATCCGCTAGCATGGAGAGGGACCGGATCTC
	Sept9MDFOR	CTCCATCCTGGAGCAGGATCGCCGGAAGCCATGAAGC
	Sept9IDREV	TGCTCCAGGATGGAGTATCCCCACGTAGCCGAAGTCC
	BamHIsfGFPREV	TAGATCCGGTGGATCCTTACTTGTACAGCTCATCCATGCC
SEPT9_i3Gmut-msfGFP	NheISept9_i3FOR	CGTCAGATCCGCTAGCATGGAGAGGGACCGGATCTC
	Sept9i1H530DFOR	GGTACCATCGAAGTTGAAAAACACCACAGATTGTGAGTTTGCCTACCTGCGG
	Sept9i1W520AREV	AAC TTCGATGGTACCCGCC TTGGTCTTCC TCCAAGGATCC
	BamHIsfGFPREV	TAGATCCGGTGGATCCTTACTTGTACAGCTCATCCATGCC
msfGFP-SEPT7Gmut1	NhelmsfGFPFOR	CGTCAGATCCGCTAGCATGGTGTCCAAGGGCGAG
	Sept7H260DFOR2	GGTGTGCTGAAGTTGAAAATGGTGAAGATTGTGATTTACAATCCTAAGAAATATGTTGATAAGAACACAC
	Sept7W250AREV2	AAC TTCAGCAACACCCG CAGGATACTGCCTTCTCTGACC
	BamHISept7i1REV	TAGATCCGGTGGATCCTTAAAAGATCTTCCCTTTCTTCTTGTTCCTTTCCAAGG
msfGFP-SEPT7Gmut2	NhelmsfGFPFOR	CGTCAGATCCGCTAGCATGGTGTCCAAGGGCGAG
	Sept7H260DFOR	TGAAGATTGTGATTTACAATCCTAAGAAATATGTTGATAAGAACACAC
	Sept7H260DREV	AAATCACAATCTTACCATTTTCAACTTACAGAACACC
	BamHISept7i1REV	TAGATCCGGTGGATCCTTAAAAGATCTTCCCTTTCTTCTTGTTCCTTTCCAAGG
mApple-SEPT7Gmut2	NhelmsfGFPFOR	CGTCAGATCCGCTAGCATGGTGTCCAAGGGCGAG
	msfGFPSept7i1FOR	GATGAGCTGTACAAGTCGGTCAGTCCGAGATCCG
	mAppleREV	CTTGTACAGCTCGTCCATGCCGCGG
	BamHISept7i1REV	TAGATCCGGTGGATCCTTAAAAGATCTTCCCTTTCTTCTTGTTCCTTTCCAAGG
SEPT9_i3-12-mEos3.2	NheISept9_i3FOR	CGTCAGATCCGCTAGCATGGAGAGGGACCGGATCTC
	Sept9linker12REV	CACCACCTCCAGAGCCACCGCCACCCATCTCTGGGGCTTCTGGCTC
	12-mEos3.2FOR	GGCTCTGGAGGTGGTGGTCTCCTCGGAAGTCCGATTAAGCCAGACATGAAG
	BamHImEos3.2REV	TAGATCCGGTGGATCCTTATCGTCTGGCATTGTACAGC
SEPT9_i3-12-mEos3.2-CAAX	NheISept9_i3FOR	CGTCAGATCCGCTAGCATGGAGAGGGACCGGATCTC
	Sept9linker12REV	CACCACCTCCAGAGCCACCGCCACCCATCTCTGGGGCTTCTGGCTC
	12-mEos3.2FOR	GGCTCTGGAGGTGGTGGTCTCCTCGGAAGTCCGATTAAGCCAGACATGAAG
	BamHICAAXmEos3.2	TAGATCCGGTGGATCCTTAGGAGAGCACACACTTGCAGCTCATGCAGCCGGGGCCACTCTCATCAGGAGGGTTCAGCTTCTGCTGGCATTGTACAGC

GFP10/GFP11 fusions in pTRIP TRE Bi (wild-type)		
Construct name	Primer name	Primer sequence
SEPT2-14-GFP11_SEPT2-14-GFP10	NdelhSept2FOR – InF	AATTCACCGGTCATATGATGTCTAAGCAACAACCAACTCAGTTTATAAATCC
	XbaIGFP11hSept2REV – InF	ATTATGATCCTCTAGATTAGGAGGCGTCGGTGATGCCGGCGGCGGTCACGTACTCCAGCAGCACCATGTGG TCGCGCTTCTCTCGGAGGACCCACCACCTCCAGAGCCACCGCCACCATCGATCACATGGTGGCCGAGA GC
	SacIIhSept2FOR – InF	TTCGAGCTCGGTACCGCGGATGTCTAAGCAACAACCAACTCAGTTTATAAATCC
	NheIGFP10hSept2REV_InF	GGAACTATTAGCTAGCTTAGTTTCAGGTCCTTGCTCAGGATGGTCTGGGTGGACAGGTAGTGGTCTCGGGC AGGTCGCCATTCCGGAGGACCCACCACCTCCAGAGCCACCGCCACCATCGATCACATGGTGGCCGAGA GC
GFP11-14-SEPT7_GFP10-14-SEPT7	NdelGFP11FOR	CATATACATATGATGGAGAAGCGCGACCACATGG
	XbaISept7i1REV	AATCCTCTAGATTAAGATCTTCCCTTTCTTCTTGTCTTTTCCAAGG
	SacIIGFP10FOR	CATATACCGCGGATGGCGACCTGCCCGAC
	NheISept7i1REV	AATCCGCTAGCTTAAAGATCTTCCCTTTCTTCTTGTCTTTTCCAAGG
SEPT9_i1-14-GFP11_SEPT9_i1-14-GFP10	NdelSept9i1FOR	CATATACATATGATGAAGAAGTCTTACTCAGGAGGCACGC
	XbaIGFP11REV	AATCCTCTAGATTAGGAGGCGTCGGTGATGCCG
	SacIISept9i1FOR	AGATCCCCCGGATGAAGAAGTCTTACTCAGGAGGCACG
	NheIGFP10REV	AATCCGCTAGCTTAGTTTCAGGTCCTTGCTCAGGATGGTC
GFP11-14-SEPT7_SEPT9_i1-14-GFP10	NdelGFP11FOR	CATATACATATGATGGAGAAGCGCGACCACATGG
	XbaISept7i1REV	AATCCTCTAGATTAAGATCTTCCCTTTCTTCTTGTCTTTTCCAAGG
	SacIISept9i1FOR	AGATCCCCCGGATGAAGAAGTCTTACTCAGGAGGCACG
	NheIGFP10REV	AATCCGCTAGCTTAGTTTCAGGTCCTTGCTCAGGATGGTC
SEPT9_i3-14-GFP11_SEPT9_i3-14-GFP10	NdelSept9i3FOR	CATATACATATGATGGAGAAGGACCGGATCTCAGC
	XbaIGFP11REV	AATCCTCTAGATTAGGAGGCGTCGGTGATGCCG
	SacIISept9i3FOR	CATATACCGCGGATGGAGAGGACCGGATCTCAG
	NheIGFP10REV	AATCCGCTAGCTTAGTTTCAGGTCCTTGCTCAGGATGGTC
GFP11-14-SEPT7_SEPT9_i3-14-GFP10	NdelGFP11FOR	CATATACATATGATGGAGAAGCGCGACCACATGG
	XbaISept7i1REV	AATCCTCTAGATTAAGATCTTCCCTTTCTTCTTGTCTTTTCCAAGG
	SacIISept9i3FOR	CATATACCGCGGATGGAGAGGACCGGATCTCAG
	NheIGFP10REV	AATCCGCTAGCTTAGTTTCAGGTCCTTGCTCAGGATGGTC
GFP10/GFP11 fusions in pTRIP TRE Bi (mutants)		
Construct name	Primer name	Primer sequence
SEPT2 NCmut-14-GFP11_SEPT2 NCmut-14-GFP10	NdelhSept2FOR – InF	AATTCACCGGTCATATGATGTCTAAGCAACAACCAACTCAGTTTATAAATCC
	XbaIGFP11hSept2REV – InF	ATTATGATCCTCTAGATTAGGAGGCGTCGGTGATGCCGGCGGCGGTCACGTACTCCAGCAGCACCATGTGG TCGCGCTTCTCTCGGAGGACCCACCACCTCCAGAGCCACCGCCACCATCGATCACATGGTGGCCGAGA GC
	SacIIhSept2FOR – InF	TTCGAGCTCGGTACCGCGGATGTCTAAGCAACAACCAACTCAGTTTATAAATCC
	NheIGFP10hSept2REV_InF	GGAACTATTAGCTAGCTTAGTTTCAGGTCCTTGCTCAGGATGGTCTGGGTGGACAGGTAGTGGTCTCGGGC AGGTCGCCATTCCGGAGGACCCACCACCTCCAGAGCCACCGCCACCATCGATCACATGGTGGCCGAGA GC
GFP11-14-SEPT7 Gmut1_GFP10-14-SEPT7 Gmut1	NdelGFP11Sept7i1FOR - InF	AATTCACCGGTCATATGATGGAGAAGCGCGACCACATGGTGTCTGAGTACGTGACCGCCCGCCGCGCAT CACCAGCCCTCCATCGATGGTGGCGGTGGCTCTGGAGGTGGTGGTCTCCGGAATGTCGGTCAGTGCG AGATCC
	XbaISept7i1REV – InF	ATTATGATCCTCTAGATTAAGATCTTCCCTTTCTTCTTGTCTTTTCC
	SacIIGFP10Sept7i1FOR - InF	TTCGAGCTCGGTACCGCGGATGGCGACCTGCCGACGACCACTACCTGTCCACCAGACCATCCTGAG CAAGGACCTGAACATCGATGGTGGCGGTGGCTCTGGAGGTGGTGGTCTCCGGAATGTCGGTCAGTGCG AGATCC
	NheISept7i1REV – InF	GGAACTATTAGCTAGCTTAAAGATCTTCCCTTTCTTCTTGTCTTTTCC
SEPT9_i1 NCmut-14-GFP11_SEPT9_i1 NCmut-14-GFP10	NdelSept9i1FOR – InF	AATTCACCGGTCATATGATGAAGAAGTCTTACTCAGGAGGCACG
	XbaIGFP11Sept9REV - InF	ATTATGATCCTCTAGATTAGGAGGCGTCGGTGATGCCGGCGGCGGTCACGTACTCCAGCAGCACCATGTGG TCGCGCTTCTCTCGGAGGACCCACCACCTCCAGAGCCACCGCCACCATCGATCACATCTGGGGCTTCTG GC
	SacIISept9i1FOR – InF	TTCGAGCTCGGTACCGCGGATGAAGAAGTCTTACTCAGGAGGCACG

	NheI GFP10Sept9REV - InF	GGAAC TATTAGCTAGCTTAGTT CAGGTCCTTGCTCAGGATGGTCTGGGTGGACAGGTAGTGGTCTCGGGC AGGTCGCCCATTC CGGAGGACCCACCACCTCCAGAGCCACC GCCACCATCGATCATCTCTGGGGCTTCTG GC
GFP11-14-SEPT7 Gmut1_SEPT9_i1 Gmut-14-GFP10	NdeI GFP11Sept7i1FOR - InF	AATTCACCGGTCATATGATGGAGAAGCGCGACCACATGGTGTGCTGGAGTACGTGACCGCCGCCGGCAT CACC GACGCCTCCATCGATGGTGGCGGTGGCTCTGGAGGTGGTGGTCTCCGGAATGTCGGTCAGTGCG AGATCC
	XbaI Sept7i1REV - InF	ATTATGATCCTCTAGATTA AAAAGATCTTCCCTTTCTTCTTGTCTTTTCC
	SacI Sept9i1FOR - InF	TTCGAGCTCGGTACCGCGGATGAAGAAGTCTTACTCAGGAGGCACG
	NheI GFP10Sept9REV - InF	GGAAC TATTAGCTAGCTTAGTT CAGGTCCTTGCTCAGGATGGTCTGGGTGGACAGGTAGTGGTCTCGGGC AGGTCGCCCATTC CGGAGGACCCACCACCTCCAGAGCCACC GCCACCATCGATCATCTCTGGGGCTTCTG GC
SEPT9_i3 NCmut-14-GFP11_SEPT9_i3 NCmut-14-GFP10	NdeI Sept9i3FOR - InF	AATTCACCGGTCATATGATGGAGAGGGACCGGATCTCAGC
	XbaI GFP11Sept9REV - InF	ATTATGATCCTCTAGATTAGGAGCGTCCGGTATGCCGGCGGTCACGTACTCCAGCAGCACCATGTGG TCGCCTTCTCTCCGGAGGACCCACCACCTCCAGAGCCACC GCCACCATCGATCATCTCTGGGGCTTCTG GC
	SacI Sept9i3FOR - InF	TTCGAGCTCGGTACCGCGGATGGAGAGGGACCGGATCTCAGC
	NheI GFP10Sept9REV - InF	GGAAC TATTAGCTAGCTTAGTT CAGGTCCTTGCTCAGGATGGTCTGGGTGGACAGGTAGTGGTCTCGGGC AGGTCGCCCATTC CGGAGGACCCACCACCTCCAGAGCCACC GCCACCATCGATCATCTCTGGGGCTTCTG GC
GFP11-14-SEPT7 Gmut1_SEPT9_i3 Gmut-14-GFP10	NdeI GFP11Sept7i1FOR - InF	AATTCACCGGTCATATGATGGAGAAGCGCGACCACATGGTGTGCTGGAGTACGTGACCGCCGCCGGCAT CACC GACGCCTCCATCGATGGTGGCGGTGGCTCTGGAGGTGGTGGTCTCCGGAATGTCGGTCAGTGCG AGATCC
	XbaI Sept7i1REV - InF	ATTATGATCCTCTAGATTA AAAAGATCTTCCCTTTCTTCTTGTCTTTTCC
	SacI Sept9i3FOR - InF	TTCGAGCTCGGTACCGCGGATGGAGAGGGACCGGATCTCAGC
	NheI GFP10Sept9REV - InF	GGAAC TATTAGCTAGCTTAGTT CAGGTCCTTGCTCAGGATGGTCTGGGTGGACAGGTAGTGGTCTCGGGC AGGTCGCCCATTC CGGAGGACCCACCACCTCCAGAGCCACC GCCACCATCGATCATCTCTGGGGCTTCTG GC
GFP10-14-SEPT7 Gmut2_GFP11-14-SEPT7 Gmut2	NdeI GFP11Sept7i1FOR - InF	AATTCACCGGTCATATGATGGAGAAGCGCGACCACATGGTGTGCTGGAGTACGTGACCGCCGCCGGCAT CACC GACGCCTCCATCGATGGTGGCGGTGGCTCTGGAGGTGGTGGTCTCCGGAATGTCGGTCAGTGCG AGATCC
	XbaI Sept7i1REV - InF	ATTATGATCCTCTAGATTA AAAAGATCTTCCCTTTCTTCTTGTCTTTTCC
	SacI GFP10Sept7i1FOR - InF	TTCGAGCTCGGTACCGCGGATGGGCGACCTGCCGACGACCACTACCTGTCCACCCAGACCATCCTGAG CAAGGACCTGAACATCGATGGTGGCGGTGGCTCTGGAGGTGGTGGTCTCCGGAATGTCGGTCAGTGCG AGATCC
	NheI Sept7i1REV - InF	GGAAC TATTAGCTAGCTTAGTT CAGGTCCTTGCTCAGGATGGTCTGGGTGGACAGGTAGTGGTCTCGGGC AGGTCGCCCATTC CGGAGGACCCACCACCTCCAGAGCCACC GCCACCATCGATCATCTCTGGGGCTTCTG GC
SEPT9_i1-14-GFP10_GFP11-14-SEPT7 Gmut2	NdeI GFP11Sept7i1FOR - InF	AATTCACCGGTCATATGATGGAGAAGCGCGACCACATGGTGTGCTGGAGTACGTGACCGCCGCCGGCAT CACC GACGCCTCCATCGATGGTGGCGGTGGCTCTGGAGGTGGTGGTCTCCGGAATGTCGGTCAGTGCG AGATCC
	XbaI Sept7i1REV - InF	ATTATGATCCTCTAGATTA AAAAGATCTTCCCTTTCTTCTTGTCTTTTCC
SEPT9_i3-14-GFP10_GFP11-14-SEPT7 Gmut2	NdeI GFP11Sept7i1FOR - InF	AATTCACCGGTCATATGATGGAGAAGCGCGACCACATGGTGTGCTGGAGTACGTGACCGCCGCCGGCAT CACC GACGCCTCCATCGATGGTGGCGGTGGCTCTGGAGGTGGTGGTCTCCGGAATGTCGGTCAGTGCG AGATCC
	XbaI Sept7i1REV - InF	ATTATGATCCTCTAGATTA AAAAGATCTTCCCTTTCTTCTTGTCTTTTCC
pnEA-vH plasmids for bacterial expression		
	Construct name	Primer name
His-TEV-SEPT2 NCmut-msfGFP, His-TEV-SEPT2 NCmut-msfGFP_SEPT6		Primer sequence
	hSept2KpnI FOR	ATCATCACAGCAGCGGTACCGGCAGCGGC GAAAACCTTTACTTCCAGGGCCATATGTCTAAGCAACAACCA ACTCAGTTTATAAATC
His-TEV-SEPT2-sfCherry2_SEPT6	NhelsfGFPREV	ATCTCCTAGGGCTAGCTCTAGACTATTAGGATCCTTACTTGTACAGCTCATCCATGCCAG
	KpnI Sept2FOR	ATCATCACAGCAGCGGTACCG
	hSept2sfCherry2	GTTGTCTCCTCCATCACATGGTGGCCGAGAGC
	sfCherry2FOR	ATGGAGGAGGACAACATGGC
BamHsfCherry2_REV	TAGACTATTAGGATCCTTAGCCGCCGGTGTGTG	