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Title

Insights into animal septins using recombinant human septin octamers with distinct SEPT9 isoforms

Francois Iv¹, Carla Silva Martins¹, Gerard Castro-Linares², Cyntia Taveneau^{3,4}, Pascale Barbier⁵, Pascal Verdier-Pinard⁶, Luc Camoin⁷, Stéphane Audebert⁷, Feng-Ching Tsai^{8,#}, Laurie Ramond¹, Alex Llewellyn¹, Mayssa Belhabib¹, Koyomi Nakazawa³, Aurélie Di Cicco³, Renaud Vincentelli⁹, Jerome Wenger¹, Stéphanie Cabantous¹⁰, Gijsje H. Koenderink^{2,8,*}, Aurélie Bertin^{3,*}, Manos Mavrakis^{1,*}

¹ Institut Fresnel, CNRS UMR7249, Aix Marseille Univ, Centrale Marseille, 13013 Marseille, France

² Department of Bionanoscience, Kavli Institute of Nanoscience Delft, Delft University of Technology, 2629 HZ Delft, The Netherlands

³ Institut Curie, Université PSL, Sorbonne Université, CNRS UMR 168, Laboratoire Physico Chimie Curie, 75005 Paris, France

⁴ ARC Centre of Excellence in Advanced Molecular Imaging, Monash University, Australia; Biomedicine Discovery Institute, Department of Biochemistry and Molecular Biology, Monash University, Clayton, Australia

⁵ Aix-Marseille Univ, CNRS, UMR 7051, Institut de Neurophysiopathologie (INP), Marseille, France

⁶ Centre de Recherche en Cancérologie de Marseille (CRCM), INSERM, Institut Paoli-Calmettes, Aix Marseille Univ, CNRS, 13009 Marseille, France

⁷ Aix-Marseille Univ, INSERM, CNRS, Institut Paoli-Calmettes, CRCM, Marseille Protéomique, Marseille, France

⁸ Department of Living Matter, AMOLF, 1098 XG Amsterdam, The Netherlands

⁹ Architecture et Fonction des Macromolécules Biologiques (AFMB), CNRS UMR7257, Aix Marseille Univ, 13009 Marseille, France

¹⁰ Centre de Recherche en Cancérologie de Toulouse (CRCT), Inserm, Université Paul Sabatier-Toulouse III, CNRS, 31037 Toulouse, France

current affiliation: Institut Curie, Université PSL, Sorbonne Université, CNRS UMR 168, Laboratoire Physico Chimie Curie, 75005 Paris, France

*Corresponding authors:

Manos Mavrakis ; ORCID ID [0000-0002-7980-1841](https://orcid.org/0000-0002-7980-1841) ; manos.mavrakis@univ-amu.fr ; mailing address: Institut Fresnel, Campus St Jérôme, 52 avenue Escadrille Normandie-Niemen, 13013 Marseille, France

Aurélie Bertin ; ORCID ID [0000-0002-3400-6887](https://orcid.org/0000-0002-3400-6887) ; aurelie.bertin@curie.fr ; mailing address: Laboratoire Physico Chimie Curie, Institut Curie, 11 rue Pierre et Marie Curie, 75005 Paris, France

Gijsje H. Koenderink ; ORCID ID [0000-0002-7823-8807](https://orcid.org/0000-0002-7823-8807) ; G.H.Koenderink@tudelft.nl ; mailing address: TU Delft, Faculty of Applied Sciences, Building 58, Van der Maasweg 9, 2629 HZ Delft, The Netherlands

key words: human septins, SEPT9 isoforms

Running Title

Recombinant human septin octamers containing SEPT9

45 **Summary Statement**

46 Iv et al. describe the first-time isolation of recombinant human septin octamers with
47 distinct SEPT9 isoforms. Octamers with either a long or a short SEPT9 isoform form
48 filaments and directly bind and cross-link actin filaments.

49

50 **Abstract**

51 Septin GTP-binding proteins contribute essential biological functions that range from the
52 establishment of cell polarity to animal tissue morphogenesis. Human septins in cells form
53 hetero-octameric septin complexes containing the ubiquitously expressed SEPT9.
54 Despite the established role of SEPT9 in mammalian development and human
55 pathophysiology, biochemical and biophysical studies have relied on monomeric SEPT9
56 thus not recapitulating its native assembly into hetero-octameric complexes. We
57 established a protocol that enabled the first-time isolation of recombinant human septin
58 octamers containing distinct SEPT9 isoforms. A combination of biochemical and
59 biophysical assays confirmed the octameric nature of the isolated complexes in solution.
60 Reconstitution studies showed that octamers with either a long or a short SEPT9 isoform
61 form filament assemblies, and can directly bind and cross-link actin filaments, raising the
62 possibility that septin-decorated actin structures in cells reflect direct actin-septin
63 interactions. Recombinant SEPT9-containing octamers will make it possible to design
64 cell-free assays to dissect the complex interactions of septins with cell membranes and
65 the actin/microtubule cytoskeleton.

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71 Introduction

72 Septins constitute a family of GTP-binding proteins conserved from algae and protists to
73 mammals (Cao et al., 2007; Momany et al., 2008; Nishihama et al., 2011; Pan et al.,
74 2007). Septins are involved in a wide range of biological processes, from the
75 establishment of cell polarity and cell division to cell-cell adhesion, cell motility, animal
76 tissue morphogenesis and infection (Fung et al., 2014; Marquardt et al., 2019; Mostowy
77 and Cossart, 2012; Weirich et al., 2008). In human pathophysiology, a role of septins has
78 been established in neuropathies, infertility and tumorigenesis (Dolat et al., 2014a;
79 Montagna et al., 2015). Despite their essential roles, how human septins organize and
80 function in cells remains much more poorly understood than for budding yeast, in which
81 septins were first discovered (Hartwell, 1971; Hartwell et al., 1970). Mammalian septins
82 are thought to associate with cell membranes (Akil et al., 2016; Bridges et al., 2016;
83 Damalio et al., 2013; Dolat and Spiliotis, 2016; Omrane et al., 2019; Tanaka-Takiguchi et
84 al., 2009; Zhang et al., 1999) like their yeast counterparts (Bertin et al., 2010; Bridges et
85 al., 2016; Bridges et al., 2014; Casamayor and Snyder, 2003). Mammalian septins
86 localize extensively to actin and microtubules in cells, for example to the ingressing
87 cytokinetic ring in dividing cells (Estey et al., 2010; Joo et al., 2007; Kim et al., 2011;
88 Kinoshita et al., 1997; Surka et al., 2002), stress fibres in interphase cells (Calvo et al.,
89 2015; Connolly et al., 2011; Dolat et al., 2014b; Joo et al., 2007; Kim et al., 2011; Kinoshita
90 et al., 2002; Kinoshita et al., 1997; Liu et al., 2014; Surka et al., 2002; Verdier-Pinard et
91 al., 2017; Xie et al., 1999; Zhang et al., 1999), and to interphase, mitotic spindle, and
92 intercellular bridge microtubules (Bowen et al., 2011; Nagata et al., 2004; Nagata et al.,
93 2003; Spiliotis et al., 2008; Spiliotis et al., 2005; Surka et al., 2002; Verdier-Pinard et al.,
94 2017). Mammalian septin association with membranes as well as with the actin and
95 microtubule cytoskeleton has made it difficult to dissect how they function, and at the
96 same time raises the intriguing possibility that septins mediate cytoskeleton-membrane
97 cross-talk.

98 Studies of native and recombinant septins isolated from budding yeast (Bertin et al., 2008;
99 Farkasovsky et al., 2005; Frazier et al., 1998; Garcia et al., 2011; Versele and Thorner,
100 2004), *Drosophila* (Field et al., 1996; Huijbregts et al., 2009; Mavrakis et al., 2014), *C.*
101 *elegans* (John et al., 2007), and mammalian cell lines and tissues (Hsu et al., 1998; Kim
102 et al., 2011; Kinoshita et al., 2002; Sellin et al., 2011; Sirajuddin et al., 2007) have
103 established that septins form heteromeric complexes, with each septin present in two
104 copies, forming a palindrome. Phylogenetic analysis has classified human septins in four
105 homology groups, namely the SEPT2 group (SEPT1, 2, 4, and 5), SEPT6 group (SEPT6,
106 8, 10, 11, and 14), SEPT7 group (SEPT7), and SEPT3 group (SEPT3, 9, and 12)
107 (Kinoshita, 2003) (see Materials and methods for nomenclature). Native human septins
108 isolated from cells exist in the form of stable hexamers and octamers (Kim et al., 2011;
109 Sellin et al., 2011; Sellin et al., 2014). Hexamers are composed of septins from the
110 SEPT2, SEPT6, SEPT7 groups, while octamers contain additional septins from the
111 SEPT3 group (Fig. 1A).

112 A well-documented feature of septins is that purified septin heteromeric complexes self-
113 assemble into filaments (Valadares et al., 2017). Whether all native septin pools are
114 filamentous, and how septin function is linked to the relative distributions of hexamers
115 and octamers and their polymerization capacity within cells are not known. The most
116 convincing evidence that septins form filaments *in vivo*, and that septin function depends
117 on their ability to assemble into filaments, comes from budding yeast (Bertin et al., 2012;
118 Byers and Goetsch, 1976; McMurray et al., 2011; Ong et al., 2014; Rodal et al., 2005).
119 A powerful tool for studying septin assembly and function has been the use of
120 recombinant septin complexes. Earlier studies using recombinant mammalian septin
121 complexes have combined septins from two or more species, most likely for pragmatic
122 reasons. Mouse SEPT2 was combined with human SEPT6 and SEPT7 (Kinoshita et al.,
123 2002; Mavrakis et al., 2014; Sirajuddin et al., 2007), or with human SEPT6, SEPT7 and
124 SEPT3 (DeRose et al., 2020), and mouse SEPT2 was also combined with human SEPT6
125 and rat SEPT7 (Bai et al., 2013). There are currently no studies showing whether these
126 specific species-related differences affect septin function. Still, taking into account that
127 these differences lie in exposed residues in the very N- or/and C-terminal extensions (Fig.
128 1A,B), or within exposed loops in the GTP-binding domain, and given how poorly we
129 understand the factors that impact animal septin assembly and function, there is a clear
130 need to produce septin complexes with full-length septins from one species, notably
131 human septin octamers containing SEPT2, SEPT6, SEPT7, and SEPT9.
132 SEPT9 is the only septin from the SEPT3 group whose expression is ubiquitous across
133 human tissues, with SEPT3 and SEPT12 being neuron- and testis-specific, respectively
134 (Cao et al., 2007; Connolly et al., 2011; Hall et al., 2005). *Sept9* gene knockout in mice is
135 embryonic lethal (Fuchtbauer et al., 2011), and a large body of literature has implicated
136 SEPT9 in diverse human cancers (Dolat et al., 2014a; Montagna et al., 2015). There are
137 five SEPT9 isoforms (SEPT9_i) differing in the length and composition of the N-terminal
138 extension preceding the GTP-binding domain (Connolly et al., 2014; McIlhatton et al.,
139 2001) (Fig. 1B). Distinct SEPT9 isoforms can have different functions, as reported for
140 cytokinesis and cancer cell migration (Estey et al., 2010; Verdier-Pinard et al., 2017).
141 Despite its importance in mammalian development and human pathophysiology,
142 biochemical and biophysical studies of SEPT9 have been limited to the use of monomeric
143 SEPT9 and fragments thereof (Bai et al., 2013; Dolat et al., 2014b; Nakos et al., 2019;
144 Smith et al., 2015), thus not recapitulating its native assembly into hetero-octameric
145 complexes (Sellin et al., 2011; Sellin et al., 2014). Multiple studies have documented
146 promiscuity in septin-septin interactions in the absence of their physiologically relevant
147 binding partners, affecting the availability of specific structural elements for interactions
148 with other septins or interacting proteins (Castro et al., 2020; Valadares et al., 2017). The
149 necessity to study septins in the context of their native heteromeric complexes is
150 highlighted by the increasing number of structural studies of the factors governing the
151 molecular specificity that determines the correct pairing of septins during complex
152 assembly (Kumagai et al., 2019; Rosa et al., 2020; Sala et al., 2016).

153 The N-terminal extension in the long SEPT9 isoforms (SEPT9_i1, SEPT9_i2 and
154 SEPT9_i3) is of considerable size (~27-kDa, i.e. three-quarters of the size of the GTP-
155 binding domain) making these isoforms the longest, in terms of the number of residues,
156 of all human septins. Given that the long SEPT9 isoforms differ only in the composition
157 of their N-terminal 25, 18 and 7 residues, respectively (Fig. 1B and Fig. S1B for SEPT9_i1
158 and SEPT9_i3), it is intriguing that they all associate with actin stress fibres in cells,
159 whereas only SEPT9_i1 associates with microtubules (Nagata et al., 2004; Nagata et al.,
160 2003; Surka et al., 2002). Different cell types express different sets of SEPT9 isoforms,
161 with some cell types expressing specific long SEPT9 isoforms, and others lacking
162 altogether long SEPT9 isoforms (Burrows et al., 2003; Sellin et al., 2014; Verdier-Pinard
163 et al., 2017). Hereditary neuralgic amyotrophy (HNA), a rare neuropathy, has been
164 mapped to missense mutations and duplications in the large N-terminal extension shared
165 by the long SEPT9 isoforms (Collie et al., 2010; Hannibal et al., 2009; Kuhlenbaumer et
166 al., 2005; Landsverk et al., 2009). Understanding SEPT9 function thus necessitates the
167 isolation of recombinant septin octamers bearing distinct SEPT9 isoforms.

168 To enable studies of SEPT9 function in the context of its physiological assembly into
169 hetero-octamers, we established a protocol that enabled, for the first time, the isolation of
170 recombinant human septin octamers containing distinct SEPT9 isoforms (Fig. 1B). A
171 combination of biochemical and biophysical assays confirmed the octameric nature of the
172 isolated octamers in solution, and also provided evidence for SEPT2 occupying the end
173 positions in the octamer. Fluorescence and electron microscopy showed that recombinant
174 octamers containing either a long or a short SEPT9 isoform form higher-order filament
175 assemblies. As a first step towards the reconstitution of recombinant SEPT9-containing
176 octamers with known physiological interactors, we examined their interactions with actin
177 filaments. Reconstitution studies showed that octamers with either a long or a short
178 SEPT9 isoform directly bind and cross-link actin filaments, raising the possibility that
179 septin-decorated actin bundles in cells reflect direct actin-septin interactions. Biochemical
180 and biophysical reconstitution studies of recombinant octamers containing distinct SEPT9
181 isoforms with physiological septin interactors, such as membranes and microtubules,
182 promise to provide a powerful complementary approach to cell and animal model studies
183 of septin organization and function.

184

185 **Results**

186

187 **A two-tag purification scheme yields stoichiometric recombinant human septin** 188 **octamers containing distinct SEPT9 isoforms**

189 To isolate octamers containing either a long SEPT9 isoform, in particular SEPT9_i1 and
190 SEPT9_i3, or octamers containing a short SEPT9 isoform, SEPT9_i5 (Fig. 1B), we
191 combined the pET-MCN (pET Multi-Cloning and expressioN) series as a septin co-
192 expression system (Diebold et al., 2011) with a two-tag purification scheme. We used two
193 bicistronic vectors: one vector co-expressing SEPT2 and SEPT6, the other one SEPT7

194 and SEPT9_i (Fig. S1A). To minimally perturb septin complex assembly, and interactions
195 with other proteins or membranes, we chose small (1 kDa) tags, a hexahistidine (His₆)
196 tag and the eight amino-acid Strep-tag II (Fig. S1B). To isolate octamers, we tagged the
197 N-terminus of the end subunit, SEPT2, with a tobacco etch virus (TEV) protease-
198 cleavable His₆-tag, and the C-terminus of the central subunit, SEPT9_i, with a TEV-
199 cleavable Strep-tag. The use of a Strep-Tactin affinity column to capture Strep-tagged
200 SEPT9_i-containing complexes, followed by a nickel affinity column to retain the
201 SEPT9_i-containing complexes that also bear His₆-tagged SEPT2, is expected to isolate
202 SEPT2-SEPT6-SEPT7-SEPT9 complexes (Fig. 1C). We used this purification scheme to
203 isolate both nonfluorescent septin complexes and fluorescent septin complexes
204 containing SEPT2 with its C-terminus fused to monomeric superfolder GFP (msfGFP)
205 (Costantini et al., 2012; Cranfill et al., 2016; Pedelacq et al., 2006; Zacharias et al., 2002).
206 Indeed, SDS-PAGE analysis of the purification of human septin complexes containing
207 SEPT9_i1 (octamers-9_i1), SEPT9_i3 (octamers-9_i3), or SEPT9_i5 (octamers-9_i5),
208 followed by Coomassie staining, showed that our purification scheme succeeded to
209 isolate SEPT2-SEPT6-SEPT7-SEPT9 complexes (Fig. 1D). The assignment of the bands
210 to the different septins was based on Western blot analysis and mass spectrometry.
211 Western blots (Fig. S1F), tryptic peptide coverage and pseudo-absolute quantitation of
212 the mol fractions of proteins in our preps by mass spectrometry (Fig. S1G,H) showed that
213 the isolated complexes were >97% pure, intact and with SEPT2, SEPT6, SEPT7, SEPT9
214 in a 1:1:1:1 stoichiometry. We note that inverting the two columns, using batch affinity
215 resins instead of prepacked columns, or combining prepacked columns and resins, all
216 provided similar results (Fig. S1C-E).

217 Given that the purification scheme *per se* cannot distinguish between tetramers and
218 octamers, we sought to determine if the isolated SEPT2-SEPT6-SEPT7-SEPT9
219 complexes were composed of tetramers or/and octamers by blue native PAGE followed
220 by Coomassie staining (Fig. 1E). For comparison, we included recombinant human
221 SEPT2-, SEPT6-, SEPT7-containing hexamers that we isolated with the same purification
222 protocol (Fig. S1 A). Blue native PAGE has been a powerful tool in detecting the presence
223 and relative distributions of endogenous septin complexes in cell lysates, and is able to
224 resolve septin tetramers from hexamers and octamers (Sellin et al., 2014). Native PAGE
225 analysis of our hexamer and octamer preps showed bands [whose size and composition](#)
226 [were in line with the presence of intact, stoichiometric hexamers for the hexamer prep](#)
227 [and intact, stoichiometric octamers for the octamer preps](#), while providing no evidence for
228 the presence of tetramers, suggesting that the latter either do not form, or they do so
229 transiently. Our findings are consistent with SEPT9 being present in the form of stable
230 octamers in cells (Sellin et al., 2011; Sellin et al., 2014).

231 To further corroborate the presence of a stable octameric population in our preps, we
232 turned to analytical ultracentrifugation sedimentation equilibrium assays, comparing side
233 by side hexamers and SEPT9_i5-containing octamers. Sedimentation equilibrium
234 experiments provide an experimental measure of the absolute mass of proteins in solution

235 (Taylor et al., 2015) and thus a powerful means of determining the species present in our
236 preps. The obtained molecular masses from such experiments were consistent with the
237 presence of hexamers for the hexamer preps, and the presence of octamers for the
238 octamer preps, without any detectable evidence for tetrameric complexes in the octamer
239 preps (Fig. 1F). We complemented these assays with analytical ultracentrifugation
240 sedimentation velocity experiments comparing octamer-9_i1, octamer-9_i3 and octamer-
241 9_i5 preps (Fig. 1G). Sedimentation velocity assays measure the experimental
242 sedimentation coefficient of proteins in solution and are thus able to detect the presence
243 of multiple protein species. Sedimentation coefficients depend on the hydrodynamic
244 properties of proteins, and are directly proportional to their mass and translational
245 diffusion coefficient, the latter including the contribution of protein shape. To interpret the
246 obtained sedimentation coefficients and given the prediction of C-terminal coiled-coils for
247 SEPT2, SEPT6 and SEPT7 (de Almeida Marques et al., 2012; Low and Macara, 2006;
248 Sala et al., 2016) (Fig. 1A,B), we used coiled-coil modeling and homology-modeling
249 software to build models of the SEPT2-SEPT6-SEPT7-SEPT9-SEPT9-SEPT7-SEPT6-
250 SEPT2 octamers. Due to the considerable size (~27 kDa) of the N-terminal extensions in
251 the long SEPT9 isoforms (SEPT9_i1, SEPT9_i3) and thus their impact on the
252 sedimentation behavior of the respective complexes, we included these in the models.
253 Given the absence of structural homologs and given secondary structure predictions of
254 disorder for this region (using Quick2D), we modeled the N-terminal extensions of SEPT9
255 as random coils (Fig. 1H, Fig. S4A) (see Materials and methods). The short (~20-30-
256 residue) N-terminal extensions of SEPT2, 6, 7 were not included in the models. We used
257 these model structures together with the Svedberg equation and the HullRad algorithm
258 (Fleming and Fleming, 2018) which calculates hydrodynamic properties of molecules
259 from their structures, to obtain the theoretical diffusion coefficients of octamers and
260 tetramers, as well as their theoretical sedimentation coefficients (table in Fig. 1G, see
261 Materials and methods for details). The experimental sedimentation coefficients for all
262 octamers-9_i1, octamers-9_i3 and octamers-9_i5 were in excellent agreement with the
263 ones estimated from the model structures, with the sedimentation coefficient distributions
264 again providing no evidence for the presence of tetramers that are expected to sediment
265 much more slowly, with a difference of ~ 3 S.

266

267 **Single particle electron microscopy analysis of recombinant septin octamers** 268 **reveals the flexibility of N- and C-terminal extensions, and provides evidence for** 269 **SEPT2 occupying the end positions**

270 To visualize the isolated octamers, we employed single particle electron microscopy (EM)
271 of negative-stained octamer preps in a high salt buffer (300 mM KCl) to prevent septin
272 complexes from polymerizing. Low magnification EM images of negative-stained octamer
273 preps highlighted the rod-like appearance of the complexes (Fig. 2A). Single particles in
274 such fields, typically ~3-4,000 particles, were computationally aligned and classified into
275 classes with distinct features (for example, orientation, curvature, or number of subunits).

276 Each class typically contained ~50-100 particles (see Materials and methods). Fig. 2B
277 shows a gallery of class averages for octamers-9_i3. Each image is the average of all the
278 particles in a given class, and has an increased signal to noise ratio compared to the raw
279 images, which allows us to distinguish individual septin subunits within the octameric
280 complex. All class averages for octamers-9_i3 exhibited a characteristic rod shape,
281 similarly to recombinant human/mammalian septins (Mavrakis et al., 2016; Mendonca et
282 al., 2019; Sirajuddin et al., 2007) or septins isolated from mammalian cell lines and tissues
283 (Hsu et al., 1998; Kim et al., 2011; Kinoshita et al., 2002; Sellin et al., 2011; Soroor et al.,
284 2021), and in line with rod-shaped septin complexes from other species, including
285 budding yeast (Bertin et al., 2008; Frazier et al., 1998; Garcia et al., 2011; Taveneau et
286 al., 2020), *C. elegans* (John et al., 2007) and *Drosophila* (Akhmetova et al., 2015; Field
287 et al., 1996; Mavrakis et al., 2014). The class averages did not show additional densities
288 at their ends or along their sides, suggesting an intrinsic orientational flexibility in the
289 junction between the G domain and the coiled-coils of SEPT2, SEPT6 and SEPT7, whose
290 densities are averaged out in such analysis. Such flexibility for the coiled-coils, deduced
291 from the absence of electron density in the crystal structure of the SEPT2-SEPT6-SEPT7
292 trimer (Sirajuddin et al., 2007) and the absence of additional densities in single particle
293 EM of budding yeast, mammalian, *C.elegans* and *Drosophila* complexes (Bertin et al.,
294 2008; Garcia et al., 2011; John et al., 2007; Mavrakis et al., 2014; Mavrakis et al., 2016;
295 Mendonca et al., 2019; Taveneau et al., 2020), seems to be a general feature of septins.
296 Moreover, there was no density that could be assigned to the large (~27-kDa) N-terminus
297 of the long isoforms, in line with secondary structure [predictions of disorder for this region](#).
298 Class averages of SEPT2-msfGFP octamers-9_i1 (Fig. 2C) did display additional
299 densities at one or both ends of the rods (green arrowheads), indicating that the GFP-
300 tagged SEPT2 subunits occupy the termini of octamers. The same observation was made
301 with SEPT2-msfGFP-containing hexamers (Fig. 2D). The fuzzy density of the C-terminal
302 GFP and its multiple positions around SEPT2 again point to a flexible hinge region
303 between the last helix of the G-domain ($\alpha 6$ helix, Fig. 1H) and the coiled-coils of SEPT2
304 (Fig. 2E).

305

306 **Recombinant septin octamers harboring either SEPT9_i1, SEPT9_i3, or SEPT9_i5** 307 **polymerize into higher-order filament assemblies in solution**

308 In addition to SEPT2 at the ends of octamers, whose presence is determinant for octamer
309 polymerization in solution, other structural elements such as the N- or C-terminal
310 extensions could also impact septin filament assembly (Bertin et al., 2010). To test the
311 effect of the SEPT9-specific N-terminal extension on septin polymerization in solution, we
312 compared octamers containing either a long or a short SEPT9 isoform. To this end,
313 octamers-9_i1, -9_i3 and -9_i5 were either dialyzed or diluted into a low-salt buffer (50
314 mM KCl). The resulting assemblies were observed with spinning disk fluorescence
315 microscopy on PLL-PEG passivated glass using SEPT2-msfGFP containing octamers
316 (Fig. S2A-C), and examined at higher spatial resolution by negative-stain EM using

317 nonfluorescent or SEPT2-msfGFP containing octamers (Fig. 3A-C). Both octamers with
318 a long or a short SEPT9 isoform polymerized into higher-order filament assemblies.
319 Fluorescence microscopy revealed a variety of assembly morphologies: optical
320 sectioning showed that all octamers assembled into interconnected and/or branched
321 networks of straight and curved filament bundles extending along 10-50 μm in the xy
322 plane and by 10-50 μm in z (Video 1 shows a representative z-stack, and Fig. S2A-C
323 show maximum-intensity projections of z-stacks). Octamers could organize into straight
324 bundles (left panel in Fig. S2B; Fig. S2C), but also into what looked like highly convoluted
325 filamentous assemblies (Fig. S2A; right panel in Fig. S2B). Measurements of isolated
326 septin filament bundles in solution for octamers-9_i3 and -9_i5 (left panel in Fig. S2B; Fig.
327 S2C) showed that they could reach up to $\sim 5\text{-}8\ \mu\text{m}$ in length.

328 Negative-stain EM similarly revealed a variety of filamentous assemblies. Octamers-9_i1
329 could organize into isolated or interconnected straight or slightly curved bundles (Fig. 3
330 A, i-ii), and into networks of interconnected highly convoluted and ring-forming filament
331 bundles (Fig. 3A, iii-v) corresponding to the similarly convoluted filamentous assemblies
332 in fluorescence microscopy (Fig. S2A). Septin filament bundles were a few μm long and
333 $\sim 50\text{-}150\ \text{nm}$ in width (Fig. S2H). High magnifications of regions within the filament bundles
334 (red-outlined insets in Fig. 3A) revealed that bundles were made of single septin filaments
335 (blue arrowheads in Fig. 3A and measurements of septin filament width in Fig. S2H)
336 running parallel to each other, with septin filaments within bundles occasionally looking
337 paired (orange arrowheads in Fig. 3A). Given the high density of filaments and the 2D
338 projection character of negative-stain EM, we cannot conclude if these are truly paired
339 filaments like budding yeast septin filament pairs (Bertin et al., 2008); if so, these would
340 have to be more tightly paired given their [narrow interfilament spacing \(\$\sim 5\ \text{nm}\$ \)](#). Similarly
341 [narrow interfilament spacing was observed recently for paired filaments formed by human](#)
342 [septin hexamers in solution \(Leonardo et al., 2021\) and by membrane-bound *Drosophila*](#)
343 [septin hexamers \(Szuba et al., 2021\)](#). Septin filament bundles exhibited a high degree of
344 interconnectivity, with a given bundle often showing splayed ends that could connect to
345 one or more different bundles, or with septin filaments forming meshes (Fig. 3A, v). We
346 speculate that the exposed, flexible coiled-coil-containing C-terminal extensions, and
347 potentially the long N-terminal extensions of SEPT9, drive the interconnections in the
348 higher-order assemblies we observe. Octamers-9_i3 also formed filament bundles (Fig.
349 3B, i) and [what appeared to be paired filaments, again with narrower interfilament spacing](#)
350 [\(\$\sim 5\ \text{nm}\$ \)](#) than the one observed for budding yeast septin filament pairs in solution (~ 10
351 nm) (Bertin et al., 2008) (Fig. 3B, ii). Octamers-9_i3 additionally formed wheel-like
352 structures associating with the bundles (Fig. 3B, i, green arrowheads). These wheels had
353 a diameter of 20-30 nm and could correspond to two or three octamer rods connected
354 end to end [forming the circumference of the wheels](#); the interior of the wheels appeared
355 to contain electron density. Very similar-looking wheels of similar dimensions have been
356 reported for budding yeast Shs1-containing octamers, with the electron density in their
357 interior attributed to the C-terminal coiled-coils stabilizing these structures (Garcia et al.,

2011; Taveneau et al., 2020). Coiled-coils in the interior of octamer-9_i3 wheels could similarly stabilize these structures, whose formation would require substantial bending of the octamers. Octamers-9_i5 also formed filaments (Fig. 3C), though the high density in these EM samples did not allow us to deduce if these are single or intertwined/paired filaments. Our combined observations from fluorescence and electron microscopy show that recombinant octamers with either a long or a short SEPT9 isoform form higher-order filament assemblies.

For comparison, we examined human septin hexamers, mammalian septin hexamers (containing mouse SEPT2, human SEPT6 and human SEPT7 Δ N19), and *Drosophila* hexamers whose polymerization was characterized previously (Mavrakis et al., 2014; Mavrakis et al., 2016). Fluorescence microscopy and negative-stain EM of human, mammalian and *Drosophila* hexamers in a low-salt buffer (Fig. S2D-G) showed that human and mammalian hexamers organized in a very similar manner into straight and curved filament bundles made of single and possibly paired septin filaments (Fig. S2F, G; Fig. S2H). *Drosophila* hexamers organized in characteristic needle-like bundles, as reported previously (Mavrakis et al., 2014; Mavrakis et al., 2016), which were not as heavily interconnected as their human counterparts. Human hexamer- and octamer-bundles displayed similar lengths and widths (Fig. S2H, see legend for median values).

376

377 **Recombinant octamers-9_i1, octamers-9_i3 and octamers-9_i5 bind and cross-link** 378 **actin filaments in solution**

379 Septins are thought to interact with actin filaments either indirectly via myosin-II (Joo et al., 2007), or directly. The possibility of direct interactions between septin hexamers and actin filaments was raised by reconstitution assays showing that recombinant mammalian and *Drosophila* hexamers bind and cross-link actin filaments into bundles (Mavrakis et al., 2014). Given that in cells both long isoforms, SEPT9_i1 and SEPT9_i3, and the short isoform SEPT9_i5 associate with the actin cytoskeleton (Connolly et al., 2011; Dolat et al., 2014b; Kim et al., 2011; Surka et al., 2002; Verdier-Pinard et al., 2017), we sought to test if recombinant octamers-9_i1, -9_i3 and -9_i5 have the capacity to directly bind and cross-link actin filaments, as hexamers do, and if so, whether the presence of a specific SEPT9 isoform makes any difference.

389 To this end, we used spinning disk fluorescence microscopy to image dilute solutions (1
390 μ M) of single actin filaments on PLL-PEG passivated glass, after spontaneous
391 polymerization of purified rabbit muscle G-actin in the presence or absence of
392 nonfluorescent or SEPT2-msfGFP-containing octamers-9_i1, -9_i3 and -9_i5 (Fig. 4A-E).
393 In the absence of octamers, fluorescence microscopy showed isolated fluctuating single
394 actin filaments, as expected (Fig. 4A and Video 2). In the presence of 0.3 μ M SEPT2-
395 msfGFP-containing octamers-9_i1 (Fig. 4C), octamers-9_i3 (Fig. 4D), or octamers-9_i5
396 (Fig. 4E), fluorescence microscopy revealed actin filaments cross-linked into straight and
397 curved bundles, in a very similar manner for all three types of octamers, and very similarly
398 to cross-linking induced by human, mammalian and *Drosophila* hexamers (Fig. S3A-C

399 and (Mavrakis et al., 2014)). Actin filament cross-linking was observed for septin
400 concentrations of 20-30 nM and above, with identical results obtained with nonfluorescent
401 septins (data not shown). The images shown in Fig. 4C-E were captured typically a few
402 hours into actin polymerization or after overnight incubation, and show the coexistence of
403 straight and curved actin filament bundles, either isolated ones or bundles connected to
404 each other forming networks. Thicker actin bundles, corresponding to a brighter signal in
405 the actin channel, fluctuated very little, suggesting that they were rigid, whereas thinner
406 actin bundles and single actin filaments, emanating from the ends or the sides of bundles,
407 or connecting neighboring bundles, were freely fluctuating (Video 3). Septins
408 systematically colocalized with the actin bundles, indicative of their actin filament cross-
409 linking activity (right panels in Fig. 4C-E depict insets of selected red-outlined regions on
410 the left). Saturating the actin and septin channels to bring out features with weaker
411 signals, notably single actin filaments (arrowheads in the actin channel in Fig. 4C-E),
412 revealed that septins localized only to actin bundles and not to single actin filaments,
413 suggesting cooperativity in septin-actin binding. Such cooperativity has also been
414 reported for other actin filament cross-linkers (Winkelman et al., 2016).

415

416 Discussion

417 In summary, our study describes the first isolation and characterization of recombinant
418 human SEPT2-SEPT6-SEPT7-SEPT9-SEPT9-SEPT7-SEPT6-SEPT2 octamers
419 containing distinct long or short SEPT9 isoforms. The employed two-tag purification
420 scheme is rapid, taking one full day starting from bacteria lysis, works with prepacked
421 columns, batch resins, or combinations thereof thus providing flexibility, and yields ~1-3
422 mg of purified octamers (a few hundreds of microliters in the micromolar concentration
423 range) [from a starting 3.5-5 L of culture](#), enabling biochemical and biophysical
424 reconstitution studies at physiological septin concentrations. A combination of
425 biochemical and biophysical assays confirmed the octameric nature of the isolated
426 complexes in solution, and also provided evidence for SEPT2 occupying the end positions
427 in the octamer, [in agreement with recent studies on septin positioning in recombinant
428 human hexamers \(Mendonca et al., 2019\), recombinant SEPT3-containing octamers
429 \(DeRose et al., 2020\) and SEPT9-containing octamers isolated from cell lysates \(Soroor
430 et al., 2021\).](#)

431 Recombinant octamers with either a long or a short SEPT9 isoform were competent for
432 polymerization in solution, [in line with studies of SEPT9-containing octamers isolated from
433 cell lysates \(Soroor et al., 2021\).](#) The observation of a large variety in the morphologies
434 of filament assemblies in solution could result from several factors. In this study we
435 examined septin assembly using recombinant octamers bound to the endogenous
436 GDP/GTP in the bacterial cytoplasm, without the exogenous addition of nucleotide during
437 cell lysis or post-purification; it is conceivable that regulation of the GDP/GTP state of
438 septins in cells regulates their higher-order assembly (Weems and McMurray, 2017). A
439 further important element is that interactions of septins with membranes, accessory

440 proteins, or the actin/microtubule cytoskeleton could influence their assembly in cells. Our
441 observations lead us to speculate that, in the absence of any other interacting surface or
442 protein, higher-order septin assembly is dominated by the exposed, flexible coiled-coil-
443 containing C-terminal extensions and/or the N-terminal extensions, resulting in the high
444 plasticity and polymorphism we observe. The potentially disordered N-terminal
445 extensions of SEPT9 could conceivably become structured (Fig. S4D-F) in the presence
446 of interacting surfaces and binding partners, for example, cell membranes and the
447 actin/microtubule cytoskeleton, resulting in a narrower range of assembly geometries.
448 The filamentous assemblies we observe in solution could reflect higher-order septin
449 filamentous assemblies that have been observed in the cytosol of cells upon perturbation
450 of interacting partners, for example, upon disruption of actin stress fibres (Joo et al., 2007;
451 Kim et al., 2011; Kinoshita et al., 2002; Kinoshita et al., 1997; Xie et al., 1999).

452 Octamers isolated from mammalian cell lysates bearing short SEPT9 isoforms were more
453 frequently observed as bundled filaments while the ones bearing long SEPT9 isoforms
454 were frequently single filaments (Soroor et al., 2021). There are two main differences
455 between the recombinant human septin octamers isolated in our study and octamers
456 immunopurified from cell lysates that could explain differences in their higher-order
457 filament assemblies. First, recombinant octamers have a defined septin composition (in
458 this case containing exclusively SEPT2, SEPT6, SEPT7, SEPT9), whereas
459 immunopurified octamers using tagged SEPT9 as a bait can contain additional septins
460 (for example, SEPT11 in (Soroor et al., 2021)). Second, differences in post-translational
461 modifications (for example septin phosphorylation and SUMOylation) in the different
462 expression systems can have a major impact on higher-order filament assembly (Estey
463 et al., 2013; Ribet et al., 2017). Understanding how specific septins contribute to higher-
464 order filament assembly and how post-translational modifications regulate human septin
465 assembly constitute challenges for future studies.

466 Recombinant human octamers-9_i1, -9_i3 and -9_i5 all shared the capacity to cross-link
467 actin filaments. The similarities with the actin filament cross-linking capacity of human
468 hexamers raise the possibility that SEPT9, in the context of an octameric complex, does
469 not contribute to actin filament cross-linking, but we cannot exclude such a contribution
470 (Dolat et al., 2014b; Smith et al., 2015). In the latter case, the contribution of SEPT9 to
471 actin filament cross-linking seems to be indistinguishable from that of the other septins in
472 the complex. Future studies of how short and long SEPT9 isoforms differ with respect to
473 their interactions with actin filaments will help provide insights into their different functional
474 contributions in cells. Our observations raise the possibility that septin-decorated actin
475 structures such as stress fibres and the cytokinetic ring reflect direct interactions between
476 actin filaments and hexameric or/and octameric septin complexes.

477 Given the importance of SEPT9 in mammalian physiology and disease, the isolation of
478 recombinant human septin octamers bearing distinct SEPT9 isoforms will facilitate
479 studies of SEPT9 in the physiological context of its assembly into hetero-octamers.
480 Septins engage in multiple interactions, making it difficult to dissect their function in the

481 complex environment of the cell. Cell-free reconstitution studies with SEPT9-containing
482 octamers and candidate interacting partners thus provide a powerful complementary
483 approach to cellular and animal model studies for exploring human septin function.

484

485 **Figure legends**

486

487 **Figure 1. Isolation and characterization of recombinant human octamers containing**
488 **distinct SEPT9 isoforms. (A-B)** Schematic representation of a mammalian septin
489 showing the conserved GTP-binding (G) domain flanked by N- and C-terminal extensions.
490 There is experimental evidence that the N-terminal extension, at least for some septins,
491 is intrinsically disordered (Garcia et al., 2006). With the exception of SEPT3 group
492 septins, the C-terminal extension is predicted to contain a coiled-coil (A). Schematics in
493 (B) depict the human septins used in this study, their size indicated by the very C-terminal
494 residue number. Residue numbers at the start and end of the G-domains correspond to
495 the start of the $\alpha 0$ helices and the end of the $\alpha 6$ helices, respectively. Residue numbers
496 right after the isoform-specific sequences for SEPT9_i1 and SEPT9_i3 indicate the start
497 of their shared long N-terminal extension. The last 28 residues of this extension constitute
498 the N-terminal extension of the short isoform, SEPT9_i5. Color-coded spheres depicting
499 the different subunits throughout the manuscript are shown next to the respective septins.
500 The freehand line preceding the SEPT9 G domain of the long isoforms depicts their large
501 N-terminal extension. **(C)** Schematic overview of the two-tag affinity purification scheme
502 for isolating stoichiometric SEPT9-containing octamers. Upon septin co-expression in the
503 bacteria cytoplasm, septins are expected to form stable hexamers and octamers (Kim et
504 al., 2011; Sellin et al., 2011; Sellin et al., 2014). Other hypothetical homo- and hetero-
505 subcomplexes could also form (Kim et al., 2012; Rosa et al., 2020; Valadares et al., 2017).
506 A first Strep-tag affinity column isolates all Strep-tagged SEPT9 complexes ("S" for Strep-
507 tag). A second nickel affinity step further isolates His₆-tagged SEPT2-containing
508 complexes ("H" for His₆-tag), thus purifying SEPT2-SEPT6-SEPT7-SEPT9 complexes.
509 **(D)** SDS-PAGE analysis of the purification of human septin octamers containing
510 SEPT9_i1 (left, 8mer-9_i1), SEPT9_i3 (middle, 8mer-9_i3) and SEPT9_i5 (right, 8mer-
511 9_i5). Coomassie-stained gels show fractions eluting from the Strep-tag affinity column,
512 from the nickel affinity column and after the final dialysis step. Molecular weight markers
513 are shown on the left; the same markers were used in all gels. The 8mer-9_i1 and 8mer-
514 9_i3 complexes shown are nonfluorescent, whereas the 8mer-9_i5 complexes shown
515 contain msfGFP-tagged SEPT2. The identification of the bands corresponding to the
516 different septins was based on mass spectrometry and Western blot analysis (Fig. S1F).
517 See Materials and methods for the theoretical and apparent molecular masses. **(E)**
518 Purified recombinant hexamers (6mer), 8mer-9_i1 and 8mer-9_i3 analyzed by blue native
519 PAGE, followed by Coomassie-staining. Molecular weight markers are shown on the left.
520 The apparent molecular masses for the recombinant 6mer and 8mer-9_i1 and -9_i3 are
521 in line with the molecular masses of native hexamers and octamers isolated from human

522 cell lysates (Sellin et al., 2014) (see also Materials and methods). [The theoretical sizes](#)
523 [of SEPT2-SEPT6-SEPT7 trimers and of SEPT2-SEPT6-SEPT7-SEPT9_i1/3 tetramers](#)
524 [are indicated as 3mer and 4mer, respectively, on the right.](#) The asterisk points to the
525 presence of [septin monomers/dimers and the chaperone DnaK](#) (see Materials and
526 methods). **(F and G)** Analytical ultracentrifugation of recombinant septin complexes.
527 Sedimentation equilibrium experiments (F) of 1.5 mg.mL⁻¹ of [SEPT2-msfGFP 6mer](#) (left)
528 and [SEPT2-msfGFP 8mer-9_i5](#) (right) at 11,000 rpm and 4°C. The filled circle symbols
529 show the experimental radial concentration distribution at sedimentation equilibrium and
530 the solid lines represent the best fit curves with the single-ideal species model. The
531 residuals representing the variation between the experimental data and those generated
532 by the fit are shown above the respective curves. The obtained experimental molecular
533 masses are indicated in the table below the curves. (G) shows the sedimentation
534 coefficient distributions $c(s)$ of [0.75 mg.mL⁻¹ 8mer-9_i1](#) (dashed line), of [0.5 mg.mL⁻¹](#)
535 [8mer-9_i3](#) (solid line) and of [1.0 mg.mL⁻¹ SEPT2-msfGFP 8mer-9_i5](#) (twodash line)
536 obtained from sedimentation velocity experiments at 40,000 rpm and 20°C. The
537 experimental sedimentation coefficients $s_{20,W}$ obtained are indicated in the table below
538 the curves. **(H)** Model of a human SEPT2-SEPT6-SEPT7-SEPT9_i1-SEPT9_i1-SEPT7-
539 SEPT6-SEPT2 octamer built using coiled-coil- and homology-modelling software (see
540 Materials and methods for details) [depicting the N-terminal extension of SEPT9_i1 as a](#)
541 [random coil](#). An *en face* view (left) and a side view after a 90° rotation (right) are shown.
542 Coiled-coils are shown to extend along the same axis as the $\alpha 6$ helix (arrow). The
543 generated model was used to calculate the theoretical translational diffusion coefficient,
544 D (cm².s⁻¹), and the latter to further calculate its theoretical sedimentation coefficient for
545 comparison with the experimentally obtained one (see Materials and methods for details).
546 [The N-terminal extensions of SEPT9_i1, SEPT9_i3 and SEPT9_i5 were all modeled as](#)
547 [random coils in the models used in these calculations](#) (table in G).

548
549 **Figure 2. Electron microscopy analysis of recombinant SEPT9-containing**
550 **octamers.** **(A)** Negative-stain EM image of recombinant 8mer-9_i1 at 25 nM in a high salt
551 buffer (300 mM KCl) showing the rod-like appearance of the complexes. **(B-E)** Single
552 particle EM analysis of recombinant septin complexes. Each panel shows a gallery of
553 representative class averages (~100 particles each) derived from the processing of
554 ~3,000-4,000 particles from micrographs of negative-stained complexes, as in (A) (see
555 Materials and methods for details). All class averages for 8mer-9_i3 complexes (B)
556 exhibited a rod shape with no evidence of additional densities at their ends or along their
557 sides, consistent with a high degree of flexibility for the coiled-coils of SEPT2, SEPT6 and
558 SEPT7, and in line with the absence of secondary structure prediction for the large (27-
559 kDa) N-terminus of the long SEPT9 isoforms. Class averages of SEPT2-msfGFP 8mer-
560 9_i1 (C) and of SEPT2-msfGFP 6mer (D) displayed additional densities at one or both
561 ends of the rods (green arrowheads), indicating that SEPT2 occupies the termini of 6mer
562 and 8mer. The fuzzy density of the C-terminal GFP and its multiple positions around the

563 end subunit suggest an intrinsic orientational flexibility in the junction between the G
564 domain and the coiled-coils of SEPT2. (E) shows the juxtaposition of a [model of the](#)
565 [octamer-9_i1 \(Fig. 1H\)](#), also containing SEPT2-msfGFP, to a high magnification class
566 average image of an octamer from (B). All septin complexes shown contain full-length,
567 human septins apart from (B) which depicts an example of mammalian septin octamers-
568 9_i3 containing mouse SEPT2-, human SEPT6-, human SEPT7 Δ N19 and human
569 SEPT9_i3 (see Materials and methods).

570

571 **Figure 3. *In vitro* reconstitution of septin polymerization in solution using**
572 **recombinant septin octamers with distinct SEPT9 isoforms. (A)** Negative-stain EM
573 images of higher-order filament assemblies upon polymerization of 8mer-9_i1 at low salt
574 (50 mM KCl). 8mer-9_i1 were polymerized at 1 μ M (A, i, ii) or 0.2 μ M (A, iii-v) final octamer
575 concentration. The insets show magnifications of selected regions of interest (dashed
576 rectangles in red), and highlight single septin filaments (blue arrowheads), possibly paired
577 septin filaments (orange arrowheads), and splayed filament bundles (v). **(B)** Negative-
578 stain EM images of higher-order filament assemblies upon polymerization of 8mer-9_i3
579 at low salt (50 mM KCl). 8mer-9_i3 were polymerized at 0.2 μ M final octamer
580 concentration. The insets show magnifications of selected regions of interest (dashed
581 rectangles in red), and highlight single septin filaments (blue arrowheads), paired septin
582 filaments (orange arrowheads), and wheel-like structures whose perimeter corresponds
583 to two or three octamers connected end to end (green arrowheads). **(C)** [Negative-stain](#)
584 [EM images of higher-order filament assemblies upon polymerization of 8mer-9_i5 at low](#)
585 [salt \(50 mM KCl\). 8mer-9_i5 were polymerized at 0.1 \$\mu\$ M \(C, i, ii\) or 0.25 \$\mu\$ M \(C, iii-v\) final](#)
586 [octamer concentration. Regions with single octamers \(~32 nm long\) are shown in i, ii.](#)
587 [Densely covered regions with septin filaments are shown in iii, iv: examples of filaments](#)
588 [\(blue arrowheads\) composed of three \(~96 nm long\) to nine octamers are shown with](#)
589 [yellow lines running parallel to the filaments.](#) All septin complexes shown contain full-
590 length, human septins apart from the left panel in (B) which depicts an example of
591 mammalian septin octamers-9_i3 containing mouse SEPT2-, human SEPT6-, human
592 SEPT7 Δ N19 and human SEPT9_i3 (see Materials and methods).

593

594 **Figure 4. *In vitro* reconstitution of actin filament cross-linking by recombinant**
595 **human septin octamers with distinct SEPT9 isoforms. (A-B)** Representative spinning
596 disk fluorescence images of reconstituted, fluctuating single actin filaments (A) upon
597 spontaneous polymerization of G-actin at 1 μ M on PLL-PEG-passivated glass in a flow
598 chamber constructed as shown in (B) (see Materials and methods for details). Actin
599 filaments are visualized by including AlexaFluor568-conjugated phalloidin during
600 polymerization. The time lapse sequence containing the still image shown on the left
601 panel of (A) is shown in Video 2. **(C-E)** Representative spinning disk fluorescence images
602 of reconstituted actin filaments, prepared as in (A), and polymerized in the presence of
603 8mer-9_i1 (C), 8mer-9_i3 (D), or 8mer-9_i5 (E). Actin filaments are visualized with

604 AlexaFluor568-conjugated phalloidin, and septins with SEPT2-msfGFP. Two examples
605 of large fields of view are shown for each, depicting the similar cross-linking of actin
606 filaments into actin filament bundles in the presence of all three types of octamers; only
607 actin labeling is shown. A time lapse sequence containing the still image shown on the
608 right panel of (C) is shown in Video 3. Insets on the right side of each panel show higher
609 magnifications of selected regions of interest on the left (dashed squares in red). Two
610 regions of interest (i, ii) are shown in each case, depicting both the actin (top row) and
611 septin (bottom row) signals. For each inset, actin and septin signals are shown in
612 duplicates: the first set shows the raw signals without any saturation, whereas the second
613 set, adjacent to the first one, shows both actin and septin signals after deliberate contrast
614 enhancement. The contrast-enhanced images in the actin channel saturate the actin
615 bundles, while bringing out weaker-intensity single actin filaments (black arrowheads).
616 The respective contrast-enhanced images in the septin channel show the presence of
617 septins in actin bundles, but their absence from single actin filaments. Scale bars in all
618 large fields of views, 10 μm . Scale bars in all insets, 5 μm . All images shown use an
619 inverted grayscale.

620

621 **Materials and methods**

622

623 **Plasmids and cloning**

624 We refer to the mammalian septin protein products as SEPT2, SEPT6, SEPT7, and
625 SEPT9 (SEPT9_i to denote isoforms; the longest isoform being i1, the next longest i2,
626 and so on), nonitalicized and with all letters capitalized, according to the mammalian
627 septin nomenclature established by (Hall et al., 2008; Macara et al., 2002). Human septin
628 gene symbols are italicized with all capital letters, and mouse septin gene symbols
629 italicized with their first letter capitalized. Mouse SEPT2, human SEPT6 and human
630 SEPT7 Δ N19 cDNAs were originally obtained from A. Wittinghofer (Max Planck Institute
631 of Molecular Physiology, Germany) and used for the expression and purification of
632 recombinant mammalian SEPT2-, SEPT6-, SEPT7-containing hexamers bearing TEV-
633 cleavable His₆-, N-terminally-tagged mouse SEPT2, human SEPT6 and noncleavable
634 Strep-tag-II-, C-terminally-tagged human SEPT7 Δ N19 (Fig. S2G and Fig. S3C) (Mavrakis
635 et al., 2014). Human SEPT9_i1 cDNA was a gift from C. Montagna (Albert Einstein
636 College of Medicine, USA). Human SEPT9_i3 cDNA was a gift from W. Trimble
637 (University of Toronto, Canada). Both human SEPT9_i1 and SEPT9_i3cDNAs have a
638 valine at position 576 for SEPT9_i1 and 558 for SEPT9_i3 instead of a methionine,
639 compared to the respective sequences in UNIPROTKB (identifiers Q9UHD8-1 and
640 Q9UHD8-2); a valine in that position is found in several human clones and many other
641 primates suggesting this is a polymorphism. We used the pET-MCN vectors pnEA-vH
642 (pET15b backbone) and pnCS (pCDF-DuET backbone) for subcloning (Diebold et al.,
643 2011).

644 Building on our cloning strategy for generating hexamers and with the plasmids available
 645 at the time (Mavrakis et al., 2014; Mavrakis et al., 2016), we generated initially a
 646 bicistronic pnEA-vH vector for co-expression of TEV-cleavable His₆-, N-terminally-tagged
 647 mouse SEPT2 and human SEPT6, and a bicistronic pnCS vector for co-expression of
 648 human SEPT7 Δ N19 and noncleavable Strep-tag-II-, C-terminally-tagged human
 649 SEPT9_i3. To this end, we digested human SEPT6 in pnCS with SpeI/XbaI, ligated the
 650 insert to SpeI-digested mouse SEPT2 in pnEA-vH and selected the clones with the
 651 correct insert orientation using restriction analysis. Similarly, we digested human
 652 SEPT9_i3 in pnCS with SpeI/XbaI, ligated the insert to SpeI-digested human SEPT7 Δ N19
 653 in pnCS and selected the clones with the correct insert orientation using restriction
 654 analysis. The combination of these vectors was used to produce and purify recombinant
 655 mammalian SEPT9_i3-containing octamers bearing TEV-cleavable His₆-, N-terminally-
 656 tagged mouse SEPT2, human SEPT6, human SEPT7 Δ N19 and noncleavable Strep-tag-
 657 II-, C-terminally-tagged human SEPT9_i3 (Fig. 2B; left panel in Fig. 3B).
 658 To introduce a TEV cleavage site for Strep-tagged SEPT9_i3 and also generate
 659 SEPT9_i1 and SEPT9_i5-containing octamers, and to introduce the missing N-terminal
 660 19 residues in SEPT7 (Fig. S1B), SEPT7 Δ N19 being initially erroneously annotated as
 661 full-length (Macara et al., 2002), we proceeded as follows. To generate full-length human
 662 SEPT7 (UNIPROTKB identifier Q1681-1), we linearized the pnCS plasmid with NdeI/NheI
 663 and employed seamless cloning (In-Fusion HD Cloning Plus Kit from Takara Bio, Cat. #
 664 638910) with the following primers: forward 5'-
 665 AAGGAGATATACATATGTCGGTCAGTGCAGATCCGCTGCTGCTGAGGAGAGGAG
 666 CGTCAACAGCAGCACCATGGTAGCTCAACAGAAGAACCCTTG-3' and reverse 5'-
 667 GCAGCCTAGGGCTAGCTCTAGACTATTAGGATC_cTTAAAAGATCTTCCCTTTCTTCT
 668 TGTTCTTTTCC-3'. To insert human SEPT9_i1, SEPT9_i3, and SEPT9_i5 with a TEV-
 669 cleavable C-terminal Strep-tag-II upstream of SEPT7, we linearized the SEPT7-
 670 containing plasmid with SpeI and used seamless cloning with the following primers:
 671 forward 5'-
 672 ACAATTCCCCACTAGTAATAATTTTGTTTAACTTTAAGAAGGAGATATACATATGAAG
 673 AAGTCT-3' for SEPT9_i1, forward 5'-
 674 ACAATTCCCCACTAGTAATAATTTTGTTTAACTTTAAGAAGGAGATATACATATGGAG
 675 AGG-3' for SEPT9_i3 and forward 5'-
 676 ACAATTCCCCACTAGTAATAATTTTGTTTAACTTTAAGAAGGAGATATACATATGGC
 677 CGACACCCCAG-3' for SEPT9_i5 and reverse 5'-
 678 CAAAATTACTAGTTTATTTTTCGAACTGCGGGTGGCTCCAGCCGCTGCCGCTG
 679 CCCTGGAAGTAAAGTTTTCCATCTCTGGGGCTTCTGGC-3' thus generating
 680 bicistronic pnCS vectors for co-expression of full-length human SEPT7 and human
 681 SEPT9_i (Fig. S1A,B).
 682 Mouse and human SEPT2 differ in 5 residues (I67V, S207N, S352G, S354G, Q359H, the
 683 latter residue being human), presenting 98.61% identity. To replace mouse SEPT2 with
 684 human SEPT2 in the bicistronic pnEA-vH vector for co-expression of TEV-cleavable His₆-

685 , N-terminally-tagged mouse SEPT2 and human SEPT6 (Fig. S1A,B), we linearized
686 pnEA-vH with KpnI/NheI and employed seamless cloning with the following primers:
687 forward 5'-
688 ATCATCACAGCAGCGGTACCGGCAGCGGCGAAAACCTTTACTTCCAGGGCCATAT
689 GTCTAAGCAACAACCAACTCAGTTTATAAATC-3' and reverse 5'-
690 ATCTCCTAGGGCTAGCTCTAGACTATTAGGATCCTCACACATGGTGGCCGAGAG-3'.
691 The human SEPT2 cDNA containing several restriction sites routinely used in cloning
692 (KpnI, NheI, BamHI) and to facilitate subsequent subcloning using these sites, we chose
693 to generate a synthetic human SEPT2 coding sequence (Eurofins Genomics, Germany)
694 that employs the codon usage of mouse SEPT2 (which does not contain the mentioned
695 restriction sites) except for the five codons that differ between the two species, for which
696 we used codons encoding the human residues V67, N207, G352, G354, H359.
697 To produce fluorescent octamers, we swapped SEPT2 in the dual expression vector for
698 SEPT2 with its C-terminus tagged with monomeric (V206K) superfolder GFP (msfGFP)
699 (no linker sequence). We generated a synthetic msfGFP coding sequence (Eurofins
700 Genomics, Germany), linearized the dual expression pnEA-vH plasmid with KpnI/NheI
701 and employed two-insert seamless cloning with the following primers: forward 5'-
702 ATCATCACAGCAGCGGTACCGGCAGCGGCGAAAACCTTTACTTCCAGGGCCATAT
703 GTCTAAGCAACAACCAACTCAGTTTATAAATC-3' and reverse 5'-
704 TTGGACACCACATGGTGGCCGAGAGC-3' for SEPT2, and forward 5'-
705 CCATGTGGTGTCCAAGGGCGAGGAGC-3' and reverse 5'-
706 ATCTCCTAGGGCTAGCTCTAGACTATTAGGATCCTTACTTGTACAGCTCATCCATGC
707 CCAG-3' for msfGFP.
708 To generate recombinant human hexamers bearing TEV-cleavable His₆-, N-terminally-
709 tagged human SEPT2, human SEPT6 and TEV-cleavable Strep-tag-II-, C-terminally-
710 tagged human SEPT7 (Fig. S1A,B; Fig. S2D,F; Fig. S3A) we employed a similar cloning
711 strategy. To insert human SEPT2 or human SEPT2-msfGFP in the pnEA-vH vector, we
712 linearized pnEA-vH with KpnI/NheI and employed seamless cloning with the same
713 primers used above in the context of the dual vector. To generate TEV-cleavable Strep-
714 tag-II-, C-terminally-tagged full-length human SEPT7, we linearized the pnCS plasmid
715 with NdeI/NheI and employed seamless cloning with the following primers: forward 5'-
716 AAGGAGATATACATATGTCGGTCAGTGCAGATCCGCTGCTGCTGAGGAGAGGAG
717 CGTCAACAGCAGCACCATGGTAGCTCAACAGAAGAACCTTG-3' and reverse 5'-
718 GCAGCCTAGGGCTAGCTCTAGACTATTAGGATCCTTATTTTTCGAACTGCGGGTGG
719 CTCCAGCCGCTGCCGCTGCCCTGGAAGTAAAGTTTTCAAAGATCTTCCCTTTCTT
720 CTTGTTCTTTTCC-3'. To insert human SEPT6 upstream of SEPT7, we linearized the
721 SEPT7-containing plasmid with SpeI and used seamless cloning with the following
722 primers: forward 5'-
723 ACAATTCCCCACTAGTAATAATTTGTTTAACTTTAAGAAGGAGATATACATATGGC
724 AGCGACCGATATAGC-3' and reverse 5'-
725 CAAATTACTAGTCTATTAGGATCCTTAATTTTTCTTCTTTGTCTCTCTTCAGA

726 GTCTGTGAG-3' thus generating a bicistronic pncS vectors for co-expression of human
727 SEPT6 and full-length human SEPT7.

728 Recombinant *Drosophila* hexamers bearing TEV-cleavable His₆-, N-terminally-tagged
729 DSep1, untagged or mEGFP-, N-terminally-tagged DSep2 and noncleavable Strep-tag-
730 II-, C-terminally-tagged Peanut were described previously (Fig. S2E) (Mavrakis et al.,
731 2014; Mavrakis et al., 2016). To generate *Drosophila* hexamers along the lines of the
732 human ones, i.e. with a TEV-cleavable Strep-tag for Peanut and with the C-terminus of
733 DSep1 (the human SEPT2 homolog) tagged with msfGFP (Fig. S2E; Fig. S3B), we
734 proceeded as follows. To insert DSep1-msfGFP in the pnEA-vH vector, we linearized
735 pnEA-vH with NdeI/BamHI and employed two-insert seamless cloning with the following
736 primers: forward 5'- ACTTCCAGGGCCATATGGCCGATACAAAGGGCTTTTC-3' and
737 reverse 5'- TTGGACACCTGCTGGGCCTGCATGC-3' for DSep1, and forward 5'-
738 CCAGCAGGTGTCCAAGGGCGAGGAGC-3' and reverse 5'-
739 TAGACTATTAGGATCCTTACTTGTACAGCTCATCCATGCCAG-3' for msfGFP. To
740 introduce the TEV cleavage site for Peanut in the bicistronic pncS vector for co-
741 expression with DSep2, we linearized the dual expression pncS plasmid with NcoI/NheI
742 and employed seamless cloning with the following primers: forward 5'-
743 CGCCAGAAGCCCATGGAG-3' and reverse 5'-
744 GCAGCCTAGGGCTAGCTCTAGACTATTAGGATCCTTATTTTTTCGAACTGCGGGTGG
745 CTCCAGCCGCTGCCGCTGCCCTGGAAGTAAAGGTTTTTCGAACAGACCCTTCTTTTT
746 CTTCTCCTTCTTGC-3'.

747 All primers for seamless cloning were Cloning Oligo (<60 bp) or EXTREmer (>60 bp)
748 synthesis and purification quality from Eurofins Genomics, Germany. All restriction
749 enzymes were FastDigest enzymes from Thermo Scientific. All plasmids were verified by
750 sequencing (Eurofins Genomics, Germany) after each cloning step, including the
751 midipreps used for protein production.

752

753 **Production and purification of recombinant human and *Drosophila* septin** 754 **complexes**

755 pnEA-vH and pncS plasmids were co-transformed into *E. coli* BL21(DE3), and co-
756 transformants selected on LB agar plates with carbenicillin and spectinomycin each at
757 100µg/mL. A single colony was selected to prepare an overnight preculture at 37°C with
758 LB medium containing antibiotics at 100µg/mL; the volume of the preculture was 1/50 of
759 the final culture volume. Terrific broth with antibiotics at 50µg/mL was inoculated with the
760 pre-culture and incubated at 37°C. We typically prepared 3.5-5 L of culture: the culture
761 volume per Erlenmeyer flask was 1/3 of the flask volume to allow for efficient oxygenation.
762 For nonfluorescent septins we let bacteria grow to A_{600nm} ~ 2 before inducing expression
763 with 0.5 mM IPTG for 3 h at 37°C. For fluorescent septins we let bacteria grow to A_{600nm}
764 ~ 0.6-0.8 before inducing expression with 0.5 mM IPTG for overnight expression at 17°C;
765 the incubator was protected from light with aluminum foil in this case. The culture was
766 stopped by centrifuging at 3,400 g for 15 min and 4°C, and the supernatant used to pool

767 all bacteria pellets in 50-mL Falcon tubes, which were further centrifuged at 5,000 g for
768 10 min and 4°C. Bacteria pellets were stored at -20°C until protein purification. Bacteria
769 expressing GFP-tagged septins yield yellow-greenish pellets.

770 On the day of purification we resuspended the pellet in ice-cold lysis buffer (50 mM Tris-
771 HCl pH 8, 300 mM KCl, 5 mM MgCl₂, 0.25 mg/mL lysozyme, 1 mM PMSF, cOmplete™
772 protease inhibitor cocktail (1 tablet per 50 mL), 10 mg/L DNase I, 20 mM MgSO₄) using
773 gentle agitation for 30 min at 4°C, and lysed cells on ice using a tip sonicator with 5 cycles
774 of 30 s "ON", 15 s "OFF". We typically use 100 mL of lysis buffer for a starting 3.5-5 L
775 culture. The lysate was clarified by centrifugation for 30 min at 20,000 g and 4°C, the
776 supernatant loaded on a StrepTrap HP column equilibrated with 50 mM Tris-HCl pH 8,
777 300 mM KCl, 5 mM MgCl₂ and the column washed with the same buffer. Strep-tag-II-
778 containing septin complexes were eluted with 50 mM Tris-HCl pH 8, 300 mM KCl, 5 mM
779 MgCl₂, and 2.5 mM desthiobiotin: desthiobiotin was prepared fresh right before use. All
780 fractions contained in the elution peak were collected. The pooled fractions were then
781 loaded to a HisTrap HP column equilibrated with 50 mM Tris-HCl pH 8, 300 mM KCl, 5
782 mM MgCl₂, the column washed with the same buffer, and His₆-tag-containing complexes
783 eluted with 50 mM Tris-HCl at pH 8, 300 mM KCl, 5 mM MgCl₂, and 250 mM imidazole.
784 We typically do not concentrate the protein further, thus we collect only the highest-
785 concentration peak fractions (~0.6 - 1.2 mL). Both affinity column steps were performed
786 on an ÄKTA pure protein purification system (Cytiva). To remove imidazole we either
787 performed an overnight dialysis step or used a PD-10 column, also including DTT in this
788 last step. The final elution buffer, in which septins are stored, was 50 mM Tris-HCl pH 8,
789 300 mM KCl, 5 mM MgCl₂, and 1 mM DTT. All purification steps were performed at 4°C.
790 We typically purify septin complexes in a single day (starting from cell lysis) to minimize
791 unnecessary exposure to proteases and contaminants and maintain protein integrity and
792 functionality.

793 Protein concentrations were assessed with absorbance measurements at 280 nm using
794 the calculated extinction coefficients for the respective complexes, and 20- or 50-µL
795 aliquots were flash-frozen in liquid nitrogen and stored at -80°C until further use. This
796 protocol yielded typically 0.5-1 mL of purified octamers at ~1-3 mg/mL (~2-6 µM) and 0.5-
797 1 mL of purified hexamers at ~1-3 mg/mL (~3-9 µM) from a starting 3.5 L of bacterial
798 culture. Extinction coefficients and molecular masses used for concentration conversions
799 were computed from the primary amino acid sequences using ExPASy
800 (<http://web.expasy.org/protparam/>) and considering two copies of each full-length septin,
801 tags included, and are summarized in [Table S1](#). The calculation of these parameters for
802 mammalian and *Drosophila* hexamers were described in (Mavrakis et al., 2016).

803 Septin preps were used closest to their purification and typically within 2-3 months upon
804 storage at -80°C. Septin complexes were typically used within 3-4 days upon thawing and
805 not frozen back. Electron microscopy of the purified septin complexes did not show
806 significant aggregation necessitating a gel filtration step, thus size exclusion
807 chromatography used previously (Mavrakis et al., 2014; Mavrakis et al., 2016) was not

808 included. Purified protein was examined as such for EM, whereas it was systematically
809 airfuged right before use for reconstitution assays using fluorescence microscopy to pellet
810 any remaining or formed aggregates upon freezing (see respective methods below). This
811 purification protocol was validated and is routinely used in three different labs (I. Fresnel,
812 I. Curie, TU Delft) with slight variations that do not affect the final result. For example, the
813 order of StrepTrap HP and HisTrap HP columns is inverted, or the nickel-affinity step uses
814 batch resin instead of prepacked columns. This production and purification protocol was
815 used to purify at least six preps of human septin octamers-9_i1, six preps of human septin
816 octamers-9_i3, one prep of human septin octamers-9_i5, and six preps of human septin
817 hexamers. The purification of mammalian septin octamers-9_i3, containing mouse
818 SEPT2, human SEPT6, human SEPT7 Δ N19, and human SEPT9_i3 (Fig. 2B and left
819 panel in Fig. 3B) was done as described previously (Mavrakis et al., 2014; Mavrakis et
820 al., 2016): the main difference with respect to the protocol described above is that the
821 columns were inverted, nickel affinity used as a first step, and that we employed an
822 additional gel filtration step.

823

824 **Materials and reagents for septin complex production and purification**

825 The sources and identifiers for the chemicals used for recombinant protein production
826 and purification are as follows. *E. coli* BL21(DE3) competent cells from Agilent (200131)
827 or Thermo Scientific (EC0114). Carbenicillin (for pNEA-vH selection) from SIGMA
828 (C3416), Condalab (6803), or Fisher Scientific (Fisher Bioreagents BP2648-1).
829 Spectinomycin (for pNCS selection) from Sigma (S4014) or Fisher Scientific (Fisher
830 Bioreagents BP2957-1). LB broth medium from Sigma (L3022) or Condalab (1231). LB
831 agar from Sigma (L2897) or Condalab (1083). SOC medium from Sigma (S1797) or
832 Fisher Scientific (Fisher Bioreagents BP9740). Terrific Broth from MP Biomedicals
833 (091012017) or Fisher Scientific (Fisher Bioreagents BP2468-10). IPTG from Euromedex
834 (EU0008-C). Imidazole with low absorbance at 280 nm from Fisher Scientific (Fisher
835 Chemical I/0010/53). PMSF from Sigma (78830). Lysozyme from Euromedex (5933).
836 cComplete™ Protease Inhibitor Cocktail Tablets from Sigma (Roche, 11836145001).
837 DNase I from Sigma (Roche, 10104159001). *d*-Desthiobiotin from Sigma (D1411). DTT
838 from Sigma (D0632). HisTrap HP 1 mL columns from Cytiva (17524701). StrepTrap HP
839 1 mL columns from Cytiva (28907546). 20K MWCO Slide-A-Lyzer cassettes from Thermo
840 Scientific (87735). PD-10 desalting columns from Cytiva (17085101).

841

842 **SDS-PAGE and Western blot**

843 We assessed septin prep purity and protein integrity by SDS-PAGE and Western blot.
844 Purified septin complexes were analyzed by SDS-PAGE using 4-20% precast
845 polyacrylamide gels (Mini-PROTEAN TGX Gels from Bio-Rad, 4561095), or hand-casted
846 12% or 15% Tris-glycine polyacrylamide gels, followed by staining with InstantBlue
847 Coomassie-stain (Expedeon, ISB1L) (Fig. S1C-E). Molecular weight markers were
848 Precision Plus Protein All Blue Standards from Bio-Rad (1610373) in all gels shown apart

849 from Fig. S1C (Pierce Unstained Protein MW Marker from Thermo Scientific, 26610) and
850 Fig. S1D (PageRuler Plus Prestained Protein Ladder from Thermo Scientific, 26619).
851 SDS-PAGE was performed for each septin prep.

852 Western blots were performed for 2 different preps for each nonfluorescent and SEPT2-
853 msfGFP hexamer and 2 different preps for each nonfluorescent and SEPT2-msfGFP long
854 SEPT9 isoform-containing octamer, with similar results. Gels for Western blot detection
855 were loaded with 10 ng of purified protein. Gel, transfer membrane (Immobilon-PSQ
856 membrane, Sigma ISEQ85R), filter pads and filter papers were incubated in transfer
857 buffer (25 mM Tris, 192 mM glycine, 20% methanol) for 15 minutes before assembly in
858 the Mini Trans-Blot transfer cell (Bio-Rad, 1703935). The transfer was done for 16 h at
859 4°C and at 110 mA constant current. The membrane was then blocked in a 5% w/v dry
860 nonfat milk TBS-T solution (20 mM Tris-HCl pH 7.5, 200 mM NaCl, 0.1 % v/v Tween20)
861 for 2 h under constant agitation. Primary and secondary antibodies were diluted in the
862 same blocking solution and incubated over the membrane for 60 min each under constant
863 agitation. Between antibody incubations, membranes were washed 3 times for 10 min
864 with TBS-T, the very last wash before detection only with TBS. To detect specific septins
865 in recombinant human hexamers and octamers, we used rabbit anti-SEPT2 (1:2,500,
866 Sigma, HPA018481), rabbit anti-SEPT6 (1:1,500, Santa Cruz Biotechnology, sc-20180),
867 rabbit anti-SEPT7 (1:200, Santa Cruz Biotechnology, sc-20620), rabbit anti-SEPT9
868 (1:1,500, Proteintech, 10769-1-AP), and HRP-conjugated anti-rabbit antibody (1:10,000,
869 Cytiva, NA934). Chemiluminescent detection was done with an Amersham ImageQuant
870 800 imager (Cytiva, 29399481) using Amersham ECL Select Western Blotting Detection
871 Reagent (Cytiva, RPN2235) diluted 5 times in Milli-Q water. The membrane was
872 incubated with the diluted reagent for 30 s, and washed for 10 s in TBS right before image
873 acquisition. Images were collected in time series mode every 10 s, for a total of 50 images,
874 and processed with ImageQuantTL software for molecular size calculation. In 4-20% Tris-
875 glycine gels, the apparent mass of SEPT6 was larger than its calculated one by ~3 kDa,
876 resulting in a band right above the one of SEPT7 that migrated as expected. The TEV-
877 Strep-tag-II of SEPT7 in hexamer preps adds 2.2 kDa to the SEPT7 band which thus
878 migrates much closer to SEPT6, making SEPT6 and SEPT7 bands hard to make out. All
879 SEPT9 isoforms migrated much more slowly than their calculated masses: the apparent
880 masses of the two long isoforms were larger by ~ 12-13 kDa, the one of the short isoform
881 by ~ 5 kDa. Western blot analysis of hexamer and octamer preps showed that all septins
882 were intact, with the long N-terminal extension of SEPT9 being the most sensitive to
883 proteolysis (Fig. S1F). The purity and protein integrity of septins in preps, as well as the
884 identification of protein bands in gels were corroborated by mass spectrometry analysis
885 (see respective section below).

886 [Given the sensitivity of the N-terminal extension of the long SEPT9 isoforms to](#)
887 [proteolysis, we strongly recommend that Western blots are systematically used to assess](#)
888 [SEPT9 integrity, in particular when preps are used for functional assays depending on](#)
889 [isoform-specific sequences. Great care must be taken throughout lysis and purification to](#)

890 minimize contamination from proteases. N-terminal fusions of long SEPT9 isoforms can
891 protect the N-terminal extensions from proteolysis (our data using msfGFP-SEPT9
892 fusions) and could be considered, as long as such fusions do not interfere with SEPT9
893 functionality. The presence of two copies of SEPT9 in the octamer cannot exclude that
894 one copy has an intact N-terminus while the other copy has a partially degraded one, thus
895 functional readouts remain the best way for assessing the integrity of the N-termini.

896

897 **Mass spectrometry analysis and data processing**

898 For analysis of septin hexamers and octamers (one prep for each hexamer and for each
899 long SEPT9 isoform-containing octamer), 1 µg of sample was loaded on 4–12% NuPAGE
900 Novex Bis-Tris polyacrylamide gels (Thermo Scientific, NP0322BOX) and ran for 7 min
901 at 80V to stack proteins in a single band. The gel was further stained with Imperial Protein
902 Stain (Thermo Scientific, 24615), destained in water and proteins cut from the gel. Gel
903 pieces (protein stack or cut protein bands) were subjected to in-gel trypsin digestion after
904 cysteine reduction and alkylation (Shevchenko et al., 2006). Peptides were extracted from
905 the gel and dried under vacuum. Samples were reconstituted with 0.1% trifluoroacetic
906 acid in 2% acetonitrile and analyzed by liquid chromatography (LC)-tandem MS (MS/MS)
907 using a Q Exactive Plus Hybrid Quadrupole-Orbitrap (Thermo Scientific,
908 IQLAAEGAAPFALGMBDK) online with a nanoLC UltiMate 3000 chromatography system
909 (Thermo Scientific, ULTIM3000RSLCNANO). 2 microliters corresponding to 10 % of
910 digested protein were injected in duplicate on the system. After pre-concentration and
911 washing of the sample on a Acclaim PepMap 100 C18 column (2 cm × 100 µm i.d., 100
912 Å pore size, 5 µm particle size, Thermo Scientific 164564-CMD), peptides were separated
913 on a LC EASY-Spray C18 column (50 cm × 75 µm i.d., 100 Å pore size, 2 µm particle
914 size, Thermo Scientific ES803) at a flow rate of 300 nL/min with a two-step linear gradient
915 (2-20% acetonitrile in 0.1 % formic acid for 40 min and 20-40% acetonitrile in 0.1 % formic
916 acid for 10 min). For peptide ionization in the EASY-Spray source, spray voltage was set
917 at 1.9 kV and the capillary temperature at 250°C. All samples were measured in a data-
918 dependent acquisition mode. Each run was preceded by a blank MS run in order to
919 monitor system background. The peptide masses were measured in a survey full scan
920 (scan range 375-1500 m/z, with 70 K FWHM resolution at m/z=400, target AGC value of
921 3.00×10^6 and maximum injection time of 100 ms). Following the high-resolution full scan
922 in the Orbitrap, the 10 most intense data-dependent precursor ions were successively
923 fragmented in the HCD cell and measured in Orbitrap (normalized collision energy of 27
924 %, activation time of 10 ms, target AGC value of 1.00×10^5 , intensity threshold 1.00×10^4
925 maximum injection time 100 ms, isolation window 2 m/z, 17.5 K FWHM resolution, scan
926 range 200 to 2000 m/z). Dynamic exclusion was implemented with a repeat count of 1
927 and exclusion duration of 10 s.

928 Raw files generated from mass spectrometry analysis were processed with Proteome
929 Discoverer 1.4.1.14 (Thermo Scientific) to search against the proteome reference of the
930 *Escherichia coli* protein database (4,391 entries, extracted from Uniprot in August 2020).

931 The original fasta file was populated with the sequences of the septin constructs
932 contained in the measured preps. Database search with Sequest HT was done using the
933 following settings: a maximum of two missed trypsin cleavages allowed, methionine
934 oxidation as a variable modification and cysteine carbamidomethylation as a fixed
935 modification. A peptide mass tolerance of 10 ppm and a fragment mass tolerance of 0.6
936 Da were allowed for search analysis. Only peptides with high Sequest scores were
937 selected for protein identification. False discovery rate was set to 1% for protein
938 identification.

939 To measure the relative protein abundance in septin preps we employed the Top3
940 quantitation approach based on the correlation between the sum of the three most intense
941 peptide ions of a given protein and its absolute abundance (Silva et al.,
942 2006). We divided the Top3 value of each identified protein in the protein stack by the
943 sum of all Top3 values, generating a relative Top3 abundance measure, which
944 correlates with the mol fraction of the protein. Septins constituted >97% of the total protein
945 content, with the remaining <3% including GTP cyclohydrolase, biotin carboxylase and
946 several chaperones (DnaK, DnaJ, GrpE, 60 kDa chaperonin). The results for molar
947 fractions down to 0.02% are shown in Fig. S1G for a hexamer and two octamer preps.
948 The obtained mol fractions of septins, compared with the expected ones for hexamers
949 (33%) and octamers (25%), point to the isolation of stoichiometric hexamers and
950 octamers.

951 Tryptic peptides were used to identify and assign each septin to the detected bands by
952 Coomassie staining, both for nonfluorescent and SEPT2-msfGFP hexamers and
953 octamers, as shown in Fig. 1D and Fig. S1C-E, and septin band assignment was in line
954 with the Western blot analysis (Fig. S1F). Examples of tryptic peptide coverage for
955 individual septins in recombinant hexamer, octamer-9_i1 and octamer-9_i3 preps are
956 shown in Fig. S1 H, with coverages of 82% (SEPT2), 74% (SEPT6) and 70% (SEPT7)
957 for hexamers, 82% (SEPT2), 70% (SEPT6), 69% (SEPT7) and 80% (SEPT9_i1) for
958 octamers-9_i1, and 82% (SEPT2), 85% (SEPT6), 68% (SEPT7) and 84% (SEPT9_i3) for
959 octamers-9_i3. Tryptic peptides were identified throughout the sequence of each septin,
960 including coiled-coils of all septins and the common N-terminal extension of the long
961 SEPT9 isoforms, which together with the apparent band sizes from SDS-PAGE and the
962 Western blot analysis (Fig. 1D; Fig. S1F) supports that the isolated septin complexes are
963 intact. [Mass spectrometry of cut protein bands from native gels corresponding to 6mers,
964 8mers-9_i1 and 8mers-9_i3 \(Fig. 1E\) further confirmed the presence of intact,
965 stoichiometric hexamers and octamers in the respective bands. Mass spectrometry of the
966 cut protein band from native gels indicated by an asterisk in Fig. 1E, identified SEPT2,
967 SEPT6, SEPT7, SEPT9 \(in the case of octamers\) and the chaperone DnaK in this band.](#)
968 Coomassie-stained bands other than the annotated ones in our figures were identified as
969 degradation products of septins or/and contaminants already identified in the analysis of
970 the complexes from protein stacks. Degradation band analysis from different preps
971 suggested that the coiled-coils of SEPT2 and SEPT7 and the N-terminal extension of

972 SEPT9 are most sensitive to proteolysis; the sensitivity of the latter to proteolysis was in
973 line with Western blots using antibodies against the C-terminal half of SEPT9 (Fig. S1F).
974 [The mass spectrometry proteomics data have been deposited to the ProteomeXchange](#)
975 [Consortium via the PRIDE partner repository with the dataset identifier PXD023857.](#)

976

977 **Native PAGE**

978 Native PAGE was performed on 4-16% NativePAGE Novex Bis-Tris polyacrylamide gels
979 (Thermo Scientific, BN1002BOX) following instructions from the manufacturer. Briefly,
980 two μg of recombinant septin complexes, in their elution buffer (50 mM Tris-HCl pH 8,
981 300 mM KCl, 5 mM MgCl_2 , 1 mM DTT), were diluted with water and 4x native PAGE
982 sample buffer (Thermo Scientific, BN2003) to achieve a total KCl/NaCl concentration of
983 ~ 100 mM and 1x native sample buffer, and were loaded in each gel well. Electrophoresis
984 was performed at 150 V constant voltage until the migration front had reached one third
985 of the gel, when dark cathode buffer was replaced with light anode buffer, then
986 electrophoresis was pursued at 150 V until the migration front had reached the bottom of
987 the gel. Gels were destained in 25% methanol and 10% acetic acid to eliminate most of
988 the background (Coomassie stain from running buffer), then washed twice in pure water
989 for 30 min, placed in Imperial Protein Stain (Thermo Scientific, 24615) for one hour and
990 destained in pure water overnight. Molecular weight standards were NativeMark
991 Unstained Protein Standard (Thermo Scientific, LC0725). Native PAGE was performed
992 for 2 independent preps for human hexamers and for each long SEPT9 isoform-
993 containing octamer, with similar results.

994 Hexamers migrated with an apparent size of ~ 310 kDa, in line with the calculated one
995 (292 kDa). Octamers for both long SEPT9 isoform-containing octamers migrated with
996 apparent sizes of ~ 600 kDa, thus much more slowly than their theoretical sizes (423 kDa
997 and 419 kDa, respectively), in line with reported gel filtration and density gradient
998 centrifugation experiments showing that the long SEPT9 isoform-specific N-terminal
999 extension confers a significant increase in the hydrodynamic radius slowing down
1000 octamer migration in native PAGE (Sellin et al., 2014).

1001

1002 **Modeling of human septin octamers**

1003 Models of human septin octamers were generated in order to analyze and interpret the
1004 analytical ultracentrifugation sedimentation velocity experiments. A series of structures of
1005 the GTP-binding domains (GBDs) of SEPT2, 6, 7 and 9 have been solved by X-ray
1006 crystallography (Rosa et al., 2020; Valadares et al., 2017), with some flexible loops
1007 partially missing. The homology modelling software, SWISS-MODEL (Waterhouse et al.,
1008 2018), was used to complete the existing GBD structures. To search for templates,
1009 SWISS-MODEL uses BLAST (Camacho et al., 2009) and HHblits (Steinegger et al.,
1010 2019) for related evolutionary structures matching the target sequence within the SWISS-
1011 MODEL Template Library (SMTL version 2020-12-09, last included PDB release: 2020-
1012 12-04). For each identified template, the quality of the resulting model is predicted from

1013 features of the target-template alignment, and the template with the highest quality is
1014 selected for model building using ProMod3. Coordinates which are conserved between
1015 the target and the template are copied from the template to the model. Insertions and
1016 deletions are remodeled using a fragment library. Finally, the geometry of the resulting
1017 model is regularized by using a CHARMM22/CMAP protein force field (Mackerell et al.,
1018 2004). The global and per-residue model quality is assessed using the QMEAN scoring
1019 function (Studer et al., 2020). GBD models, starting right after the end of the $\alpha 0$ helices
1020 and until the end of the $\alpha 6$ helices, were generated for SEPT2 37-306 (template PDB
1021 6UPA), SEPT6 40-308 (template PDB 6UPR), SEPT7 48-316 (template PDB 6N0B), and
1022 SEPT9 295-568 (template PDB 5CY0; numbering based on SEPT9_i1). [The lack of
1023 structural information for the short \(~20-30-residue\) N-terminal extensions of SEPT2, 6,
1024 7, including the \$\alpha 0\$ helices, prompted us not to model these regions. However, the long
1025 \(~260-280-residue\) N-terminal extension of the long SEPT9 isoforms, SEPT9_i1 and
1026 SEPT9_i3, is expected to have an important impact on the hydrodynamic properties of
1027 the complexes. The lack of structural information for this N-terminal extension, the
1028 absence of structural homologs for this region \(using Phyre2 and SWISS-MODEL\) and
1029 secondary structure predictions of disorder for this region \(using Quick2D\), prompted us
1030 to model the N-terminal extensions of SEPT9_i1 and SEPT9_i3 as random coils with
1031 Phyre 2 \(Kelley et al., 2015\), using an *ab initio* approach \(Fig.1H, Fig. S4A\). The
1032 generated model of the N-terminal extension of SEPT9_i1 as a random coil was used to
1033 isolate the short \(~30-residue\) N-terminal extension of the short SEPT9 isoform,
1034 SEPT9_i5, for modeling the latter in SEPT9_i5-containing complexes \(Fig. S4A\).
1035 Structure prediction using RaptorX, an algorithm based on distance-based protein folding
1036 powered by deep learning \(Xu, 2019\), generated alternative models of the N-terminal
1037 extensions of SEPT9_i1 \(Fig. S4D,E\) and SEPT9_i3 \(Fig. S4F\), including an extended
1038 conformation of the N-terminal extension of SEPT9_i1 \(Fig. S4D\).](#)

1039 To account for the contribution of the predicted coiled-coils in the C-terminal extensions
1040 of SEPT2, 6 and 7 to the hydrodynamic properties of the complexes and thus their
1041 sedimentation behavior, we extended the GBD models to include the C-terminal domain
1042 from the end of the $\alpha 6$ helix onwards. Delineation of coiled-coil features was based on
1043 secondary structure prediction via Quick2D (Zimmermann et al., 2018). This tool
1044 integrates secondary structure predictions from different softwares, including coiled-coil
1045 prediction via MARCOIL (Delorenzi and Speed, 2002), PCOILS (Gruber et al., 2006) and
1046 COILS (Lupas et al., 1991). The consensus sequences assigned by all three coiled-coil
1047 prediction algorithms were used for modeling coiled-coil helices with CCFold software
1048 (Guzenko and Strelkov, 2018). The resulting coiled-coils encompass residues 310-349,
1049 321-406, and 336-421 for SEPT2, 6, and 7, respectively.

1050 The models built with SWISS-MODEL and CCFold were still missing the connections
1051 between the GBDs and coiled-coils for SEPT2, 6, and 7, the ends of the C-terminal
1052 domains right after the predicted CCs, and the C-terminal domain of SEPT9 after the $\alpha 6$
1053 helix. Phyre2 (Kelley et al., 2015) was used to construct these flexible parts *ab initio* for

1054 SEPT2, 7 and 9; and by homology for the very end of SEPT6 as a structural homolog
1055 was found by the software. The different models were generated in the context of the full
1056 proteins for higher accuracy. The flexible parts linking the GBDs and coiled-coils and the
1057 remaining C-terminal features were isolated from the resulting models with PyMOL (open-
1058 source software). GBDs, coiled-coils, flexible parts, [the N-terminal extensions, and, when](#)
1059 [relevant, sfGFP \(PDB 2B3P\)](#), were then combined with PyMOL. The connections
1060 between the GBDs and the coiled-coils for SEPT6 and 7 being of different length and to
1061 allow for the aligning of coiled-coil helices of SEPT6 and 7, these connections were
1062 stretched out so that they cover the same distance between the GBDs and the start of
1063 the coiled-coils without any steric clashes. In the case of SEPT2, the 3 residues between
1064 the GBD and the coiled-coil were built directly with PyMOL.

1065 Tetrameric SEPT2-SEPT6-SEPT7-SEPT9 and octameric SEPT2-SEPT6-SEPT7-
1066 SEPT9-SEPT9-SEPT7-SEPT6-SEPT2 complexes were built by fitting the modeled
1067 structures to the crystal structure of the SEPT2-SEPT6-SEPT7 trimer (PDB 2QAG)
1068 (Sirajuddin et al., 2007). [The central SEPT9 NC interface dimer in the octamer was built](#)
1069 [by aligning the modeled monomer to the SEPT2 NC dimer of the filament present in the](#)
1070 [crystal packing of the SEPT2-SEPT6-SEPT7 trimer structure. The other subunits were](#)
1071 [subsequently assembled to the dimer SEPT9-SEPT9 by aligning the modeled monomers](#)
1072 [to the rest of the X-ray structure.](#) The PDB files of the modeled tetramers and octamers,
1073 with or without the C-terminal extensions, [and with different orientations of the CC](#)
1074 [domains](#), were then used in HullRad to determine their diffusion coefficient and estimate
1075 their sedimentation coefficient [using the full-length molecular mass of the respective](#)
1076 [complexes](#) (see section on analytical ultracentrifugation below). [The model with the](#)
1077 [extended conformation of the N-terminal extension of SEPT9_i1 \(Fig. S4C\) was used](#)
1078 [interchangeably for SEPT9_i3 for predicting the sedimentation behavior of such](#)
1079 [hypothetical complexes \(Fig. S4B\).](#)

1080

1081 **Analytical ultracentrifugation**

1082 A sedimentation velocity experiment was carried out for one prep each of octamers-9_i1
1083 (0.75 mg/mL), octamers-9_i3 (0.5 mg/mL), [and SEPT2-msfGFP octamers-9_i5 \(1.0](#)
1084 [mg/mL\)](#) at 40,000 rpm and 20°C in a Beckman Optima XL-A analytical ultracentrifuge,
1085 using 12 mm double sector centerpieces in an AN-50 Ti rotor (Beckman Coulter). Scans
1086 were acquired in continuous mode at 280 nm, in the absorbance range of 0.1 to 1. The
1087 partial specific volume of the proteins and the density and viscosity of the buffer were
1088 calculated with SEDNTERP (Laue et al., 1992). At 20°C, the calculated partial specific
1089 volume for octamers-9_i1 and -9_i3 was 0.735 mL.g⁻¹ and [for SEPT2-msfGFP octamers-](#)
1090 [9_i5 0.736 mL.g⁻¹.](#) The density and viscosity of the buffer (50 mM Tris-HCl pH 8, 300 mM
1091 KCl, 5 mM MgCl₂, 1 mM DTT [or 1 mM TCEP](#)) were 1.014 g.mL⁻¹ and 0.0102 poise,
1092 respectively. The data recorded from moving boundaries were analyzed in terms of
1093 continuous size distribution functions of sedimentation coefficient, $c(s)$, using the program

1094 SEDFIT (Schuck and Rossmann, 2000) and the apparent sedimentation coefficient at
1095 20°C in water ($S_{20,w}$) determined by peak integration.

1096 A short column sedimentation equilibrium experiment was carried out for one prep each
1097 of SEPT2-msfGFP hexamers and SEPT2-msfGFP octamers-9_i5, at 11,000 rpm in a
1098 Beckman Optima XL-A analytical ultracentrifuge, using 60 μ L of protein loading
1099 concentrations from 0.5 to 1.5 mg.mL⁻¹, in a six-channel epon charcoal-filled centerpiece
1100 in an AN-50 Ti rotor (Beckman Coulter). Septins were in 50 mM Tris-HCl pH 8, 300 mM
1101 KCl, 5 mM MgCl₂, and 1 mM TCEP. Scans were acquired at appropriate wavelengths
1102 (280 nm and 485 nm) when sedimentation equilibrium was reached at 4°C. Average
1103 molecular masses were determined by fitting a sedimentation equilibrium model for a
1104 single sedimenting solute to individual data sets with SEDPHAT.

1105 To determine the theoretical sedimentation coefficient, the PDB file of a given model
1106 (tetramer, hexamer or octamer, [including GFP in the case of SEPT2-msfGFP complexes](#),
1107 with or without coiled-coils, and with coiled-coils in different orientations) was analyzed
1108 using HullRad (Fleming and Fleming, 2018) to determine the translational diffusion
1109 coefficient, D . The estimated sedimentation coefficient, s , was then obtained using the
1110 theoretical molecular mass, M , for each complex and the Svedberg equation below, with
1111 v the partial specific volume of the protein, ρ the solvent density, R the gas constant and
1112 T the temperature:

$$s = \frac{M(1-\bar{v}\rho)D}{RT}$$

1116 The theoretical sedimentation coefficients calculated in this manner are shown in Fig. 1G,
1117 Fig. S1I [and Fig. S4B](#).

1118

1119 **Preparation of flow cells for fluorescence microscopy of *in vitro* reconstituted actin** 1120 **and septins**

1121 Microscope glass slides and coverslips were cleaned for 15 min in base-piranha solution
1122 (Milli-Q water, 30% ammonium hydroxide, 35% hydrogen peroxide at a 5:1:1 volume
1123 ratio), rinsed twice, 5 min each, with Milli-Q water in a bath sonicator, and stored in 0.1 M
1124 KOH up to one month. Right before assembling flow cells, slides and coverslips were
1125 rinsed twice, 5 min each, with Milli-Q water and dried with synthetic air. Flow cells with
1126 \sim 10 μ L channels were assembled by sandwiching \sim 2-mm-wide and \sim 2.5-cm-long strips
1127 of Parafilm between a cleaned glass slide and coverslip and melting on a hot plate at
1128 120°C (Fig. 4B). The resulting chambers were passivated by incubating for 45 min with 1
1129 M KOH, rinsing with actin polymerization buffer (see composition in the respective
1130 section), incubating for another 45 min with 0.2 mg/mL PLL-PEG, and rinsing with actin
1131 polymerization buffer (5 mM Tris-HCl pH 8, 50 mM KCl, 1 mM MgCl₂, 0.2 mM Na₂ATP, 1
1132 mM DTT). Flow cells were placed in a Petri-dish along with tissue paper soaked in water
1133 to prevent flow channels from drying during the incubation steps and until use.

1134 The sources and identifiers for the materials and chemicals are as follows. Glass slides
1135 (26x76 mm) from Thermo Scientific (AA00000102E01FST20). Glass coverslips (24x60
1136 mm) from Thermo Scientific (BB02400600A113FST0). Ammonium hydroxide solution
1137 from SIGMA (221228). Hydrogen peroxide solution from SIGMA (95299). PLL-PEG from
1138 SuSoS AG (PLL(20)-g[3.5]-PEG(2)).

1139

1140 **Sample preparation for fluorescence microscopy of *in vitro* reconstituted actin and** 1141 **septins**

1142 Lyophilized rabbit skeletal muscle G-actin was resuspended to 5 mg/mL (119 μ M) in G-
1143 buffer (5 mM Tris-HCl pH 8, 0.2 mM Na₂ATP, 0.1 mM CaCl₂, 1 mM DTT), aliquots snap-
1144 frozen in liquid nitrogen and stored at -80°C. Frozen aliquots were thawed and
1145 centrifuged for 30 min at 120,000 g in a benchtop Beckman air-driven ultracentrifuge
1146 (Beckman Coulter Airfuge, 340401) to clear the solution from aggregates. Clarified G-
1147 actin was kept at 4°C and used within 3-4 weeks.

1148 For reconstitution experiments, G-actin was diluted with G-buffer to 5 μ M, and
1149 polymerized at 1 μ M final concentration in actin polymerization buffer (5 mM Tris-HCl pH
1150 8, 50 mM KCl, 1 mM MgCl₂, 0.2 mM Na₂ATP, 1 mM DTT), additionally containing 1 mM
1151 Trolox, 2 mM protocatechuic acid (PCA), 0.1 μ M protocatechuate 3,4-dioxygenase (PCD)
1152 and 0.1% w/v methylcellulose. Trolox and the enzymatic oxygen scavenging system
1153 PCA-PCD were used to minimize photobleaching during fluorescence imaging (Cordes
1154 et al., 2009; Shi et al., 2010). Methylcellulose was used as a crowding agent to keep actin
1155 filaments close to the surface and facilitate their observation. To fluorescently label actin
1156 filaments, we polymerized G-actin in the presence of 1 μ M Alexa Fluor 568-conjugated
1157 phalloidin.

1158 For actin-septin reconstitution experiments, thawed septin aliquots were cleared for 15
1159 min at 120,000 g in a Beckman airfuge right before use. To polymerize G-actin in the
1160 presence of septins, we followed the same procedure as above, but mixed G-actin with
1161 septins, either nonfluorescent ones or GFP-labeled septins (at 20% GFP molar ratio for
1162 octamers-9_i1 and -9_i3, and 100% GFP for octamers-9_i5) to a final septin
1163 concentration of 0.3 μ M, right before polymerization. Actin and actin-septin samples were
1164 prepared with a final volume of 10 μ L, were loaded immediately into PLL-PEG-passivated
1165 flow channels upon mixing of the components to start polymerization, and flow channels
1166 were sealed with VALAP (1:1:1 vasoline:lanoline:paraffin). The contributions of KCl and
1167 MgCl₂ from the septin elution buffer were taken into account to yield the same final
1168 composition of actin polymerization buffer. Actin and actin-septin samples were typically
1169 incubated overnight at room temperature in the dark before observation. Actin-septin
1170 assays were repeated at least four times, using at least two different preps from each
1171 nonfluorescent and fluorescent hexamers, 8mers-9_i1 and 8mers-9_i3, and one prep
1172 from fluorescent 8mers-9_i5, yielding similar results.

1173 To polymerize septins in the absence of actin, we followed the same procedure as above,
1174 but replaced the G-actin solution with G-buffer. Septins were also polymerized in the

1175 absence of actin by overnight dialysis against a low-salt buffer (50 mM Tris-HCl pH 8, 50
1176 mM KCl, 5 mM MgCl₂, 1 mM DTT) at 4°C, then loaded into PLL-PEG-passivated flow
1177 channels in the presence of 1 mM Trolox, 2 mM PCA, 0.1 μM PCD and 0.1% w/v
1178 methylcellulose, and sealed as described above for observation. Septins were used at
1179 20% or 100% GFP molar ratio, yielding similar results. Septin polymerization assays were
1180 repeated at least five times, using at least two different preps from each nonfluorescent
1181 and fluorescent hexamers, 8mers-9_i1 and 8mers-9_i3, and one prep from fluorescent
1182 8mers-9_i5, yielding similar results.

1183 Actin-septin samples with mammalian septin hexamers (Fig. S3C) were prepared as
1184 above with the difference that septins were nonfluorescent, and fluorescent actin was
1185 Alexa Fluor 488-conjugated G-actin (10% molar ratio) as described previously (Mavrakis
1186 et al., 2014; Mavrakis et al., 2016). G-actin polymerization in this case occurred in the
1187 presence of nonfluorescent phalloidin.

1188 The sources and identifiers for proteins, materials and chemicals are as follows. Rabbit
1189 skeletal muscle G-actin from Cytoskeleton, Inc. (AKL99). Alexa Fluor 568-phalloidin from
1190 Thermo Scientific (A12380). Nonfluorescent phalloidin from Sigma (P2141).
1191 Methylcellulose from Sigma (M0512). Trolox from Sigma (238813). Protocatechuic acid
1192 from Sigma (03930590). Protocatechuate 3,4-dioxygenase from Sigma (P8279). 20K
1193 MWCO Slide-A-Lyzer MINI dialysis devices from Thermo Scientific (69590).

1194

1195 **Fluorescence microscope image acquisition and processing**

1196 Samples were imaged on an optical setup employing a confocal spinning disk unit (CSU-
1197 X1-M1 from Yokogawa) connected to the side-port of a Perfect Focus System-equipped
1198 inverted microscope (Eclipse Ti2-E from Nikon Instruments), using a Nikon Plan Apo
1199 ×100/1.45 NA oil immersion objective lens, 488- and 561-nm Sapphire laser lines
1200 (Coherent) and an iXon Ultra 888 EMCCD camera (1024×1024 pixels, 13×13 μm pixel
1201 size, Andor, Oxford Instruments), resulting in an image pixel size of 65 nm. Images were
1202 acquired with an exposure time of 0.1 s. Time-lapse sequences were acquired with a time
1203 interval of 0.5 s for a duration of 15 s. Actin filaments and actin-septin bundles were
1204 imaged close to the surface. Septin filament bundles were also found at the surface, but
1205 the extensive clusters of interconnected human septin filament bundles were observed
1206 floating in the bulk of the flow channels. To capture such clusters, z-stacks were acquired
1207 over 10-50 μm using a Δz interval of 0.5 μm. The images shown correspond to octamers-
1208 9_i1 polymerized with 20% GFP-septins (Fig. S2A), octamers-9_i3 polymerized with
1209 100% GFP-septins (Fig. S2B), octamers-9_i5 polymerized with 100% GFP-septins (Fig.
1210 S2C), hexamers polymerized with 100% GFP-septins (Fig. S2D), and *Drosophila*
1211 hexamers polymerized with 20% GFP-septins (Fig. S2E). All examples shown depict
1212 polymerization upon dilution into low salt apart from Fig. S2D (left panel) which shows
1213 polymerization upon dialysis into low salt.

1214 Images were processed with the open-source image processing software ImageJ/Fiji.

1215 Images of actin filaments and actin-septin bundles are from single planes. Images of

1216 septin filament bundles are from maximum-intensity z projections except for *Drosophila*
1217 septins, for which single planes are shown given that their bundles were typically found
1218 primarily at the surface. The contrast of all images shown was adjusted post-acquisition
1219 so that both dim and bright structures are visible. To saturate the signal in the actin
1220 bundles and make the weaker-intensity signal of single/thinner actin filaments visible, the
1221 contrast was enhanced on purpose (images labeled "contrast enhancement" in Fig. 4C-
1222 E and Fig. S3A-B). All images shown use an inverted grayscale, with bright signals
1223 appearing black in a white background.

1224 Actin-septin samples with mammalian septin hexamers (Fig. S3C) were imaged with a
1225 Nikon Apo TIRF $\times 100/1.49$ NA oil immersion objective lens mounted on an Eclipse Ti
1226 microscope (Nikon Instruments) using a 491 nm laser line and imaged with a QuantEM
1227 512SC EMCCD camera (Photometrics). Images were acquired with an exposure time of
1228 0.1 s.

1229

1230 **Transmission electron microscopy**

1231 **Negative stain electron microscopy.** 4 μ L of sample at final septin concentrations of
1232 0.01-0.02 mg/mL (~ 25 -50 nM) for high salt conditions (50 mM Tris-HCl pH 8, 300 mM
1233 KCl, 2 mM $MgCl_2$) or 0.05-0.1 mg/mL (~ 125 -250 nM) for low salt conditions (50 mM Tris-
1234 HCl pH 8, 50 mM KCl, 2 mM $MgCl_2$) were adsorbed for 30 s (for high salt conditions) to 1
1235 h in a humid chamber (for low salt conditions) on a glow-discharged carbon-coated grid
1236 (Electron Microscopy Sciences, CF300-CU). For low salt conditions, septins were
1237 polymerized by dilution into low-salt buffer and incubated for 1 h to overnight at room
1238 temperature before grid adsorption. In the case of GFP-labeled septins, septins were
1239 polymerized without mixing with nonfluorescent ones. The grids were rinsed and
1240 negatively stained for 1 min using 1% w/v uranyl formate. Images for the qualitative
1241 examination of the morphology of septin assemblies were collected using a Tecnai Spirit
1242 microscope (Thermo Scientific, FEI) operated at an acceleration voltage of 80kV and
1243 equipped with a Quemesa camera (Olympus). In addition to the EM experiments
1244 described above which were performed at I. Curie, EM was also performed at TU Delft
1245 following a similar protocol. Septins were polymerized by dilution into a low-salt buffer (25
1246 mM Tris-HCl pH 7.4, 50 mM KCl, 2.5 mM $MgCl_2$, 1 mM DTT) at a final septin concentration
1247 of 1 μ M for 1 h. The solution was then adsorbed to a glow discharged grid for 1 min,
1248 rinsed, negatively stained with 2% w/v uranyl acetate for 30 s and air dried. Images were
1249 collected with a JEM-1400plus TEM microscope (JEOL) operated at 120kV equipped with
1250 4k X 4k F416 CMOS camera (TVIPS). Septin filament bundle length and width
1251 measurements (Fig. S2H) were made with the line tool in ImageJ/Fiji, and boxplots
1252 generated in Matlab. [The narrow interfilament spacing \(\$\sim 5\$ nm\) in paired filaments](#)
1253 [mentioned in the main text refers to center-to-center spacing, and was deduced from](#)
1254 [paired filament width measurements assuming a monomer size of \$\sim 3.5\$ nm.](#) Electron
1255 microscopy was performed with at least two different preps from each nonfluorescent and
1256 fluorescent hexamers, 8mers-9_i1, 8mers-9_i3 and 8mers-9_i5. The images shown
1257 correspond to nonfluorescent octamers-9_i1 (Fig. 3A, i-ii), octamers-9_i3 (Fig. 3B) and

1258 octamers-9_i5 (Fig. 3C), SEPT2-msfGFP octamers-9_i1 (Fig. 3A, iii-v), SEPT2-msfGFP
1259 hexamers (Fig. S2F), and nonfluorescent mammalian hexamers (Fig. S2G).

1260
1261 **Two-dimensional image processing for single-particle EM images.** Images for single-
1262 particle analysis were collected using a Lab6 G2 Tecnai microscope (Thermo Scientific)
1263 operated at an acceleration voltage of 200 kV. Images were acquired with a 4k X 4k F416
1264 CMOS camera (TVIPS) in an automated manner using the EMTools software suite
1265 (TVIPS) with a pixel size of 2.13 Å and an electron dose of about 15 electrons/Å². 2D
1266 processing was carried out on septin rods incubated in high salt conditions (50 mM Tris-
1267 HCl pH 8, 300 mM KCl, 2 mM MgCl₂) to determine the integrity of the complexes and
1268 pinpoint the arrangement of septin subunits within the complex. About 100 images were
1269 collected for each septin complex for image processing. Individual particles (septin rods)
1270 were hand-picked from the images using the boxer tool from the EMAN software suite
1271 (Ludtke et al., 1999). About 20-30 particles (203x203 pixel boxes) were extracted per
1272 image. Subsequent processing was carried out using SPIDER (Frank et al., 1996). After
1273 normalization of the particles, a non-biased reference-free algorithm was used to
1274 generate 20 classes. Those classes were further used as references to pursue 2D
1275 multivariate statistical analysis. Multi-reference alignment followed by hierarchical
1276 classification involving principal component analysis was thereafter carried out to
1277 generate classes containing 50-100 particles. Each of the classes are representative of
1278 specific features within a given sample. This processing enabled us to quantify the
1279 distribution of particles in each dataset regarding the dimension of the rods as well as the
1280 presence of an additional electron density (GFP-tag). For mammalian octamers-9_i3 (Fig.
1281 2B), 4000 particles were selected with a distribution of 50% octamers, 23.7% heptamers,
1282 23.5 % hexamers, 1.4% pentamers and 1.4% tetramers. For human SEPT2-msfGFP
1283 octamers-9_i1 (Fig. 2C), 3266 particles were picked with a distribution of 57.9% octamers,
1284 32.1% heptamers and 10.1% hexamers. An additional density towards the ends of the
1285 rods was pinpointed for 46.2% of the particles. For human SEPT2-msfGFP hexamers
1286 (Fig. 2D), 2976 particles were selected with a distribution of 97.7% hexamers and 2.3%
1287 pentamers. An additional density towards the ends of the rods could be pinpointed for
1288 53.6% of the particles.

1289

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1326

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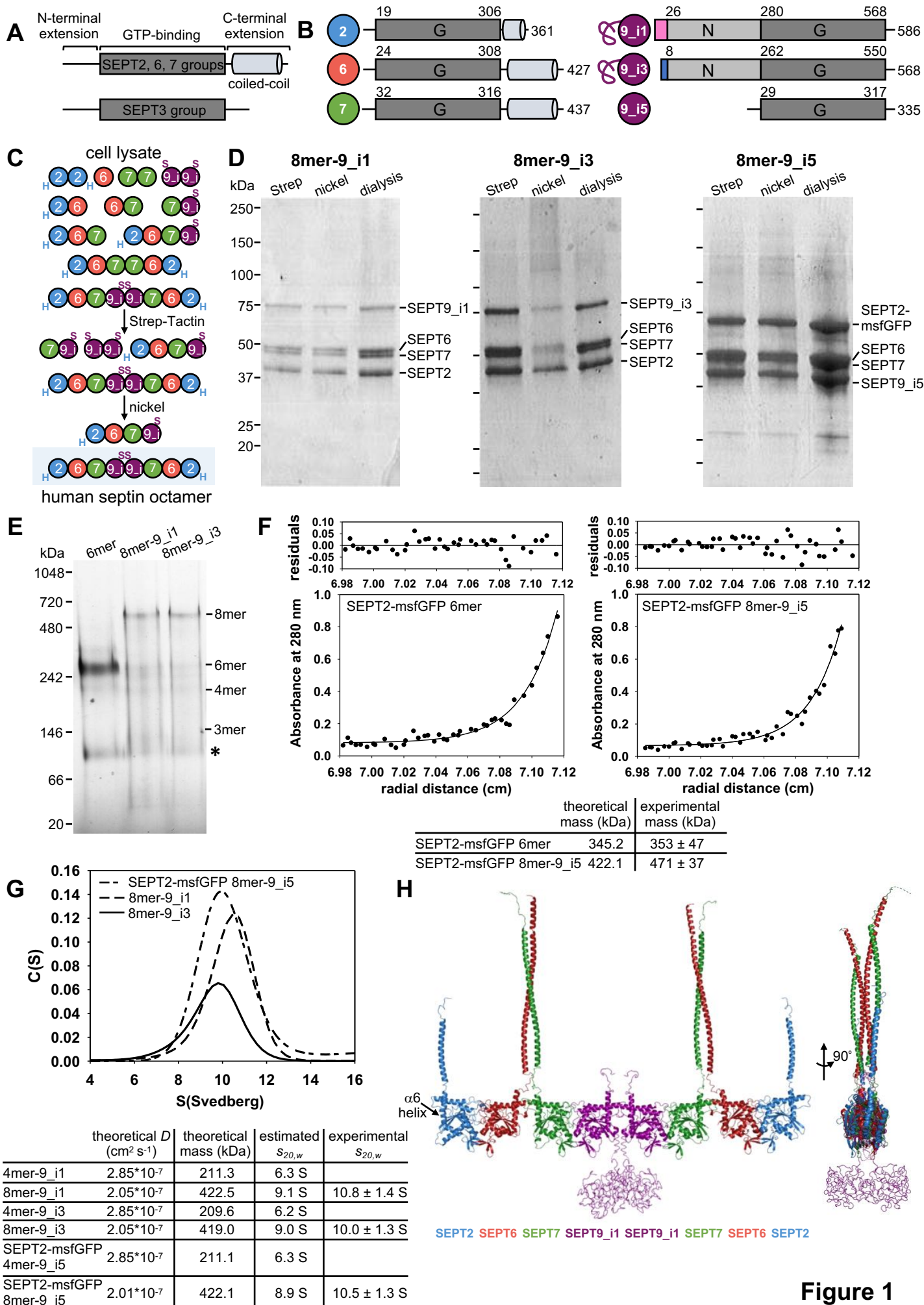


Figure 1
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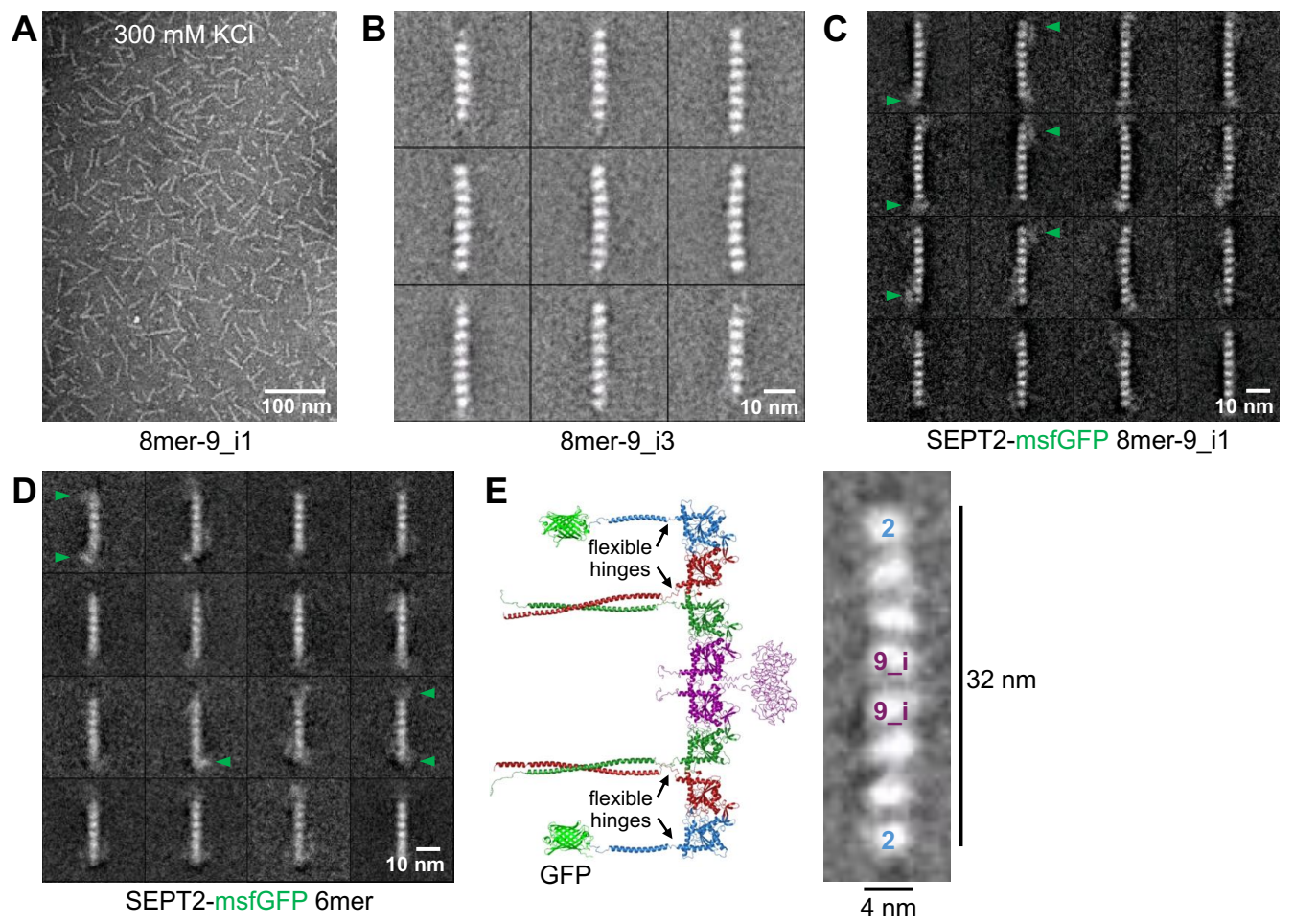
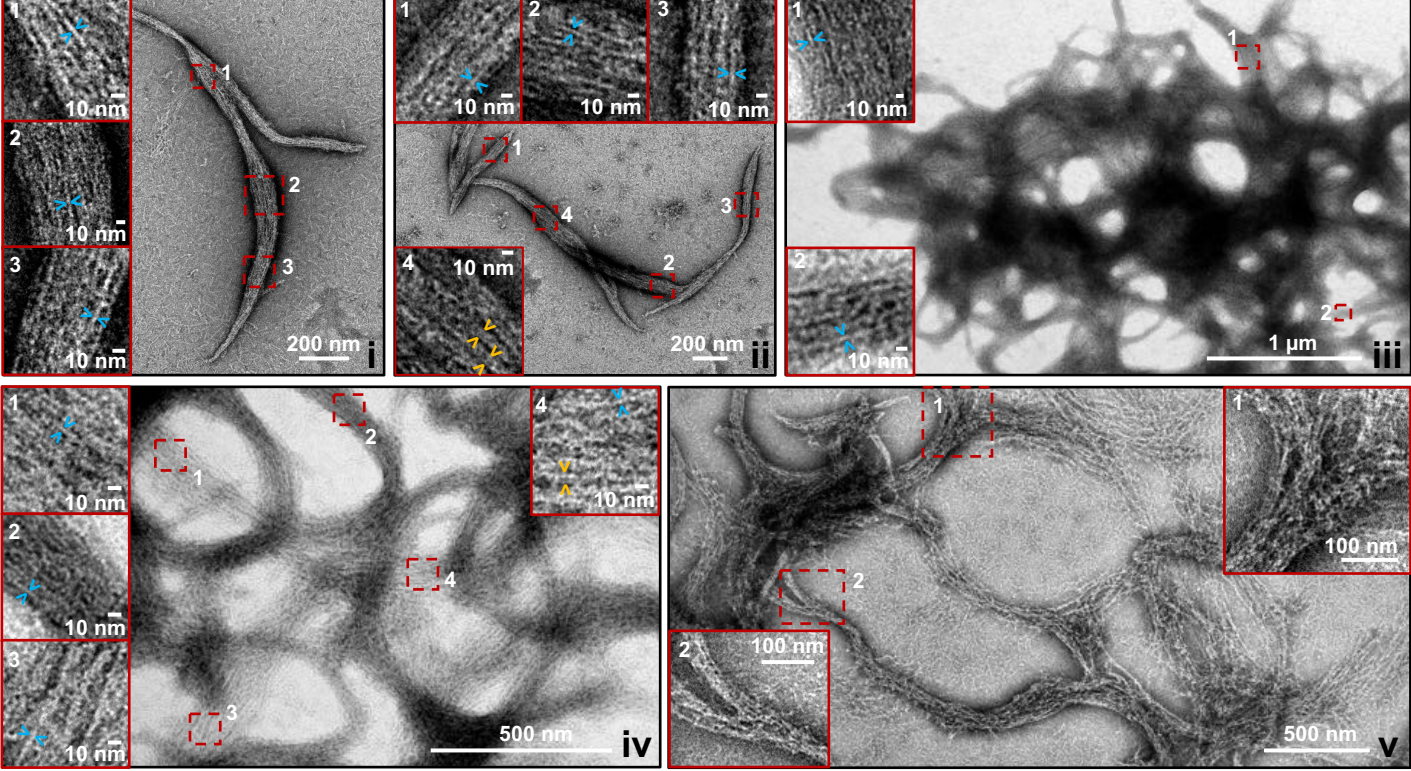
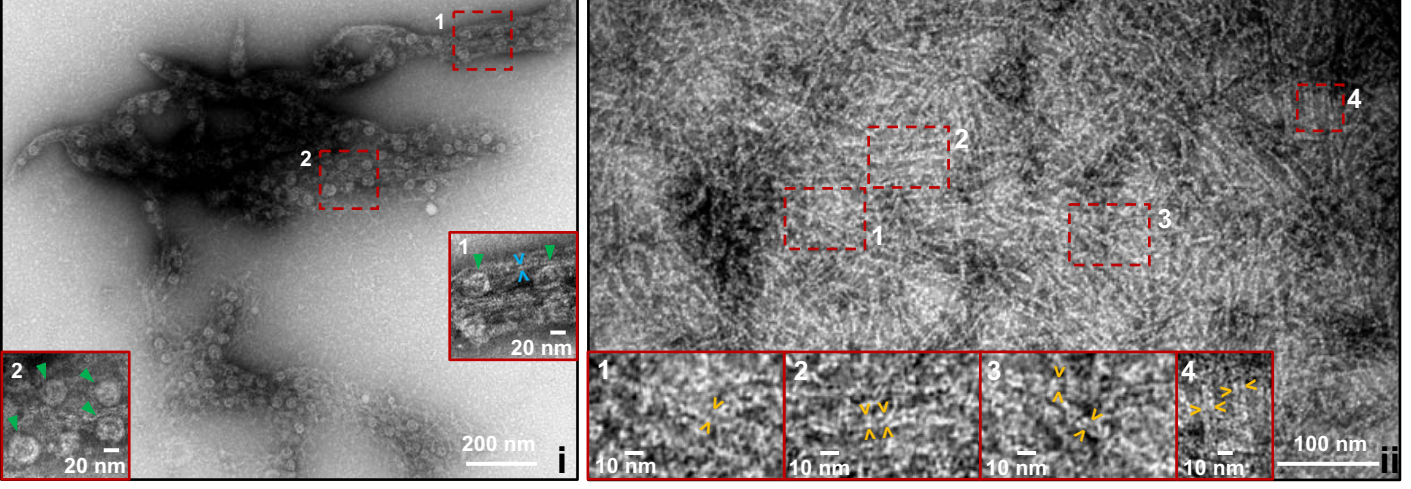


Figure 2
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A 2 6 7 9.i1 9.i1 7 6 2
808



B 2 6 7 9.i3 9.i3 7 6 2
808



C 2 6 7 9.i5 9.i5 7 6 2

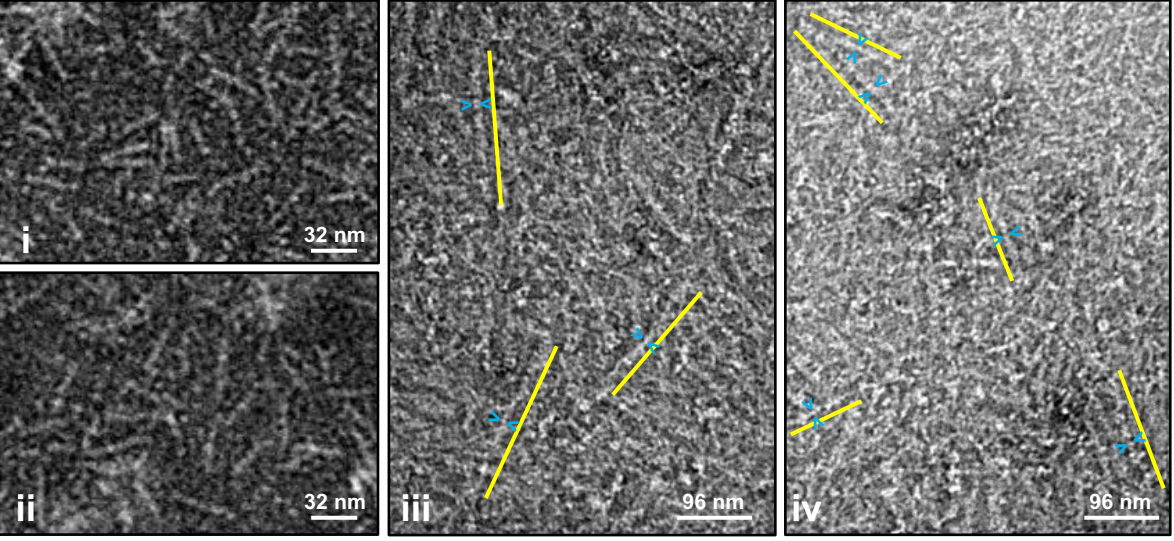


Figure 3
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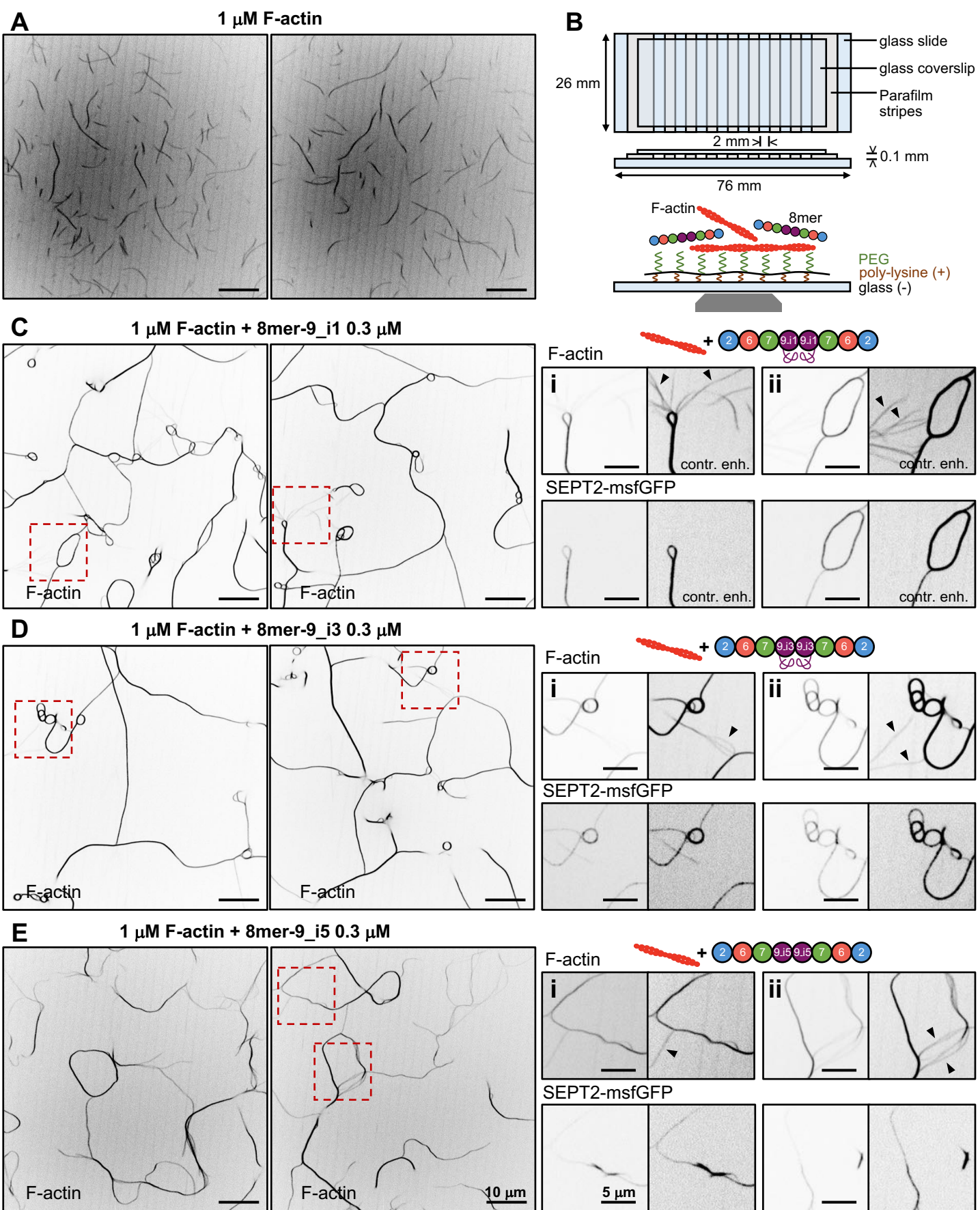


Figure 4
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Supplementary material

Fig. S1 contains details on the plasmids and septin sequences used for the isolation of recombinant human septin hexamers and octamers. Fig. S1 further contains additional data on the biochemical and biophysical characterization of septin complexes that relate to Fig. 1. Fig. S2 shows fluorescence and electron microscopy data on septin assembly from recombinant human septin octamers and recombinant human, mammalian and *Drosophila* septin hexamers, and relates to Fig. 3. Fig. S3 shows fluorescence microscopy data on septin-actin reconstitution using recombinant human, mammalian and *Drosophila* septin hexamers, and relates to Fig. 4. Fig S4 shows models of the N-terminal extensions of short and long SEPT9 isoforms, and relates to Fig. 1.

Figure S1. **Isolation and characterization of recombinant human septin octamers containing distinct SEPT9 isoforms.** **(A)** Schematic of the two sets of plasmids used for the co-expression of septins for isolating recombinant human SEPT2-, SEPT6-, SEPT7-containing hexamers (left) and recombinant human SEPT2-, SEPT6-, SEPT7-, SEPT9_i-containing octamers (right) from bacteria. SpeI and XbaI restriction sites used for subcloning are indicated (see Materials and methods for details). The end subunit (SEPT2) contains an N-terminal TEV-cleavable His₆-tag (depicted as H in the cartoons of the septin complexes), while the central subunit (SEPT7 in hexamers, or SEPT9_i in octamers) contains a C-terminal TEV-cleavable Strep-tag II. The plasmids for the production of fluorescent septin complexes used in this study differ only in that the gene coding for SEPT2 has been replaced by the one encoding SEPT2-msfGFP. **(B)** Primary sequences of the N- and C-termini of tagged septins used in the purification schemes in this study. His₆-tag, Strep-tag-II, and TEV cleavage site sequences are highlighted in light orange. Black arrowheads indicate the position of the TEV cleavage site. Asterisks point to the last amino acid of the respective septin sequence. The underlined amino acids in the C-terminus of SEPT2 are three out of the five residues that differ among the mouse and human homologs. The underlined stretch of N-terminal residues in SEPT7 was missing in previously reported plasmids (see Materials and methods for details). SEPT9 long isoform-specific sequences are highlighted in pink and cyan (see Fig. 1B). The sequence depicting the onset of the short isoform SEPT9_i5, which is also contained in the N-terminal extensions of the long isoforms, is highlighted in green. **(C-E)** SDS-PAGE analysis of the purification of mammalian (C and D) and human (E) SEPT2-, SEPT6-, SEPT7-containing hexamers. Coomassie-stained gels show fractions from the total lysate (T), supernatant (S/N), flow-through (F/T), wash (W), eluate (E), and after concentration (C), using a two-tag purification scheme employing either a nickel affinity step followed by a Strep-Tactin affinity step (C), or a Strep-Tactin affinity step followed by a nickel affinity step (D and E). Molecular weight markers are shown on the left of each gel. The identification of bands is based on mass spectrometry analysis. The asterisk in (C) points to putative His₆-tagged SEPT2 homodimers that are removed in the Strep-tag affinity step. **(F)** Purified recombinant nonfluorescent and fluorescent (SEPT2-msfGFP)

6mer, 8mer-9_i1 and 8mer-9_i3 were analyzed by SDS-PAGE, followed by Western blot (WB) with antibodies against SEPT2, SEPT6, SEPT7, and SEPT9, as indicated at the bottom of each gel (see Materials and methods for details). Molecular weight markers are shown for the first gel; the same markers were used in all gels. All septins were intact, the long N-terminal extension of SEPT9 being most sensitive to proteolysis (the asterisk points to a degradation product for SEPT9). See Materials and methods for the theoretical and apparent molecular masses. **(G-H)** Examples of mass spectrometry analysis of recombinant 6mer, 8mer-9_i1 and 8mer-9_i3 preps. Calculations of the mol fractions of septins and contaminants in the respective protein preps are shown (G) using the Top3 quantitation approach (see Materials and methods for details). The obtained mol fractions of septins, compared with the theoretical ones in 6mer (33%) and 8mer (25%), point to the isolation of stoichiometric 6mers and 8mers. Examples of tryptic peptide coverage for individual septins in recombinant 6mer, 8mer-9_i1 and 8mer-9_i3 preps (H), supporting that the isolated septin complexes are intact. **(I)** Models for octamers without coiled-coils, or with coiled-coils at 90° with respect to the $\alpha 6$ helix, pointing to the same or to opposite directions as shown on the right for a model of an octamer-9_i1, were used to calculate their theoretical sedimentation coefficients (see Materials and methods for details). The N-terminal extensions of SEPT9_i1, SEPT9_i3 and SEPT9_i5 were modeled as random coils in the models used in these calculations. The absence of coiled-coils altogether is predicted to make the complexes sediment faster by ~ 0.8 S. Coiled-coils lying on the same side tend to make complexes more compact and thus slightly accelerate sedimentation by ~ 0.1-0.5 S, whereas coiled-coils on opposite sides are predicted to slow down sedimentation by ~ 0.6-0.7 S units.

Figure S2. ***In vitro* reconstitution of septin polymerization in solution using recombinant animal septin hexamers and octamers.** **(A-C)** Representative spinning disk fluorescence images of higher-order filament assemblies upon polymerization of human 8mer-9_i1 (A), 8mer-9_i3 (B) and 8mer-9_i5 (C) after dilution into low salt conditions (50 mM KCl) at the indicated final octamer concentration. Two examples are shown for each. All images shown are maximum-intensity projections and use an inverted grayscale. **(D-E)** Representative spinning disk fluorescence images of higher-order filament assemblies upon polymerization of human SEPT2-, SEPT6-, SEPT7-containing hexamers (D) and *Drosophila* DSep1-, DSep2-, Peanut-containing hexamers (E) after dilution into low salt conditions (50 mM KCl) at the indicated final concentration. Two different *Drosophila* hexamers are shown: hexamers labeled with mEGFP-DSep2 (left panel in E) and hexamers labeled with DSep1-msfGFP (right panel in E). Two examples are shown for each type of hexamer. *Drosophila* hexamers organize in straight needle-like bundles, in line with previous reports (Mavrakis et al., 2014; Mavrakis et al., 2016). The freehand line preceding the G domain of Peanut in the hexamer cartoon above the images depicts its large N-terminal extension. Images in (D) are maximum-intensity projections. All images shown use an inverted grayscale. **(F)** Negative-stain EM images

of higher-order filament assemblies upon polymerization of human 6mer at 0.2 μM and at low salt (50 mM KCl). The insets show magnifications of selected regions of interest (dashed rectangles in red), and highlight single septin filaments (blue arrowheads), paired septin filaments (orange arrowheads), and splayed filament bundles (i, iii). **(G)** Negative-stain EM of higher-order filament assemblies upon polymerization of mouse SEPT2-, human SEPT6-, human SEPT7 Δ N19-containing hexamers at low salt (50 mM KCl) and at 1 μM (i, ii) or 0.5 μM (iii). The insets show magnifications of selected regions of interest (dashed rectangles in red), and highlight single septin filaments (blue arrowheads), paired septin filaments (orange arrowheads), and splayed filament bundles (ii). **(H)** Box plots showing the distribution of septin filament bundle lengths (left), septin filament bundle widths (middle) and septin filament widths within bundles (right), measured from electron micrographs, and comparing human 6mer- (red-filled circles) and 8mer-9_i1 (blue-filled circles) filament assemblies. The data points are plotted on top of the respective box plots. 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 most extreme data points not considered outliers, and the outliers are plotted individually using the 'x' symbol. The number of measurements in each box plot, ordered from left to right, is $n = 58, 83, 229, 69, 30, 28$. The respective median values are 1.9 μm , 1.3 μm , 78 nm, 67 nm, 4.6 nm, and 3.9 nm.

Figure S3. ***In vitro* reconstitution of actin filament cross-linking by recombinant animal septin hexamers.** **(A-C)** Representative spinning disk fluorescence images of reconstituted actin filaments, polymerizing in the presence of human SEPT2-, SEPT6-, SEPT7-containing hexamers (A), *Drosophila* DSep1-, DSep2-, Peanut-containing hexamers (B), and mouse SEPT2-, human SEPT6-, human SEPT7 Δ N19-containing hexamers (C), prepared as in Fig.4C-D. (A-B) Actin filaments are visualized with AlexaFluor568-conjugated phalloidin, and septins with SEPT2-msfGFP (human) or DSep1-msfGFP (*Drosophila*). Two examples of large fields of view are shown for each, depicting the similar cross-linking of actin filaments into actin filament bundles in the presence of both types of hexamers; only actin labeling is shown. Insets on the right side of each panel show higher magnifications of selected regions of interest on the left (dashed squares in red). Two regions of interest (i, ii) are shown in each case, depicting both the actin (top row) and septin (bottom row) signals. For each inset, actin and septin signals are shown in duplicates: the first set shows the raw signals without any saturation, whereas the second set, adjacent to the first one, shows both actin and septin signals after deliberate contrast enhancement. The contrast-enhanced images in the actin channel saturate the actin bundles, while bringing out weaker-intensity single actin filaments (black arrowheads). The respective contrast-enhanced images in the septin channel show the presence of septins in actin bundles, but their absence from single actin filaments. Scale bars in all large fields of views, 10 μm . Scale bars in all insets, 5 μm . (C) Actin filaments are visualized with Alexa Fluor 488-G-actin and septins are

nonfluorescent. Three examples of large fields of view are shown, depicting the similar cross-linking of actin filaments into actin filament bundles. Scale bars in all large fields of views, 10 μm . All images shown use an inverted grayscale.

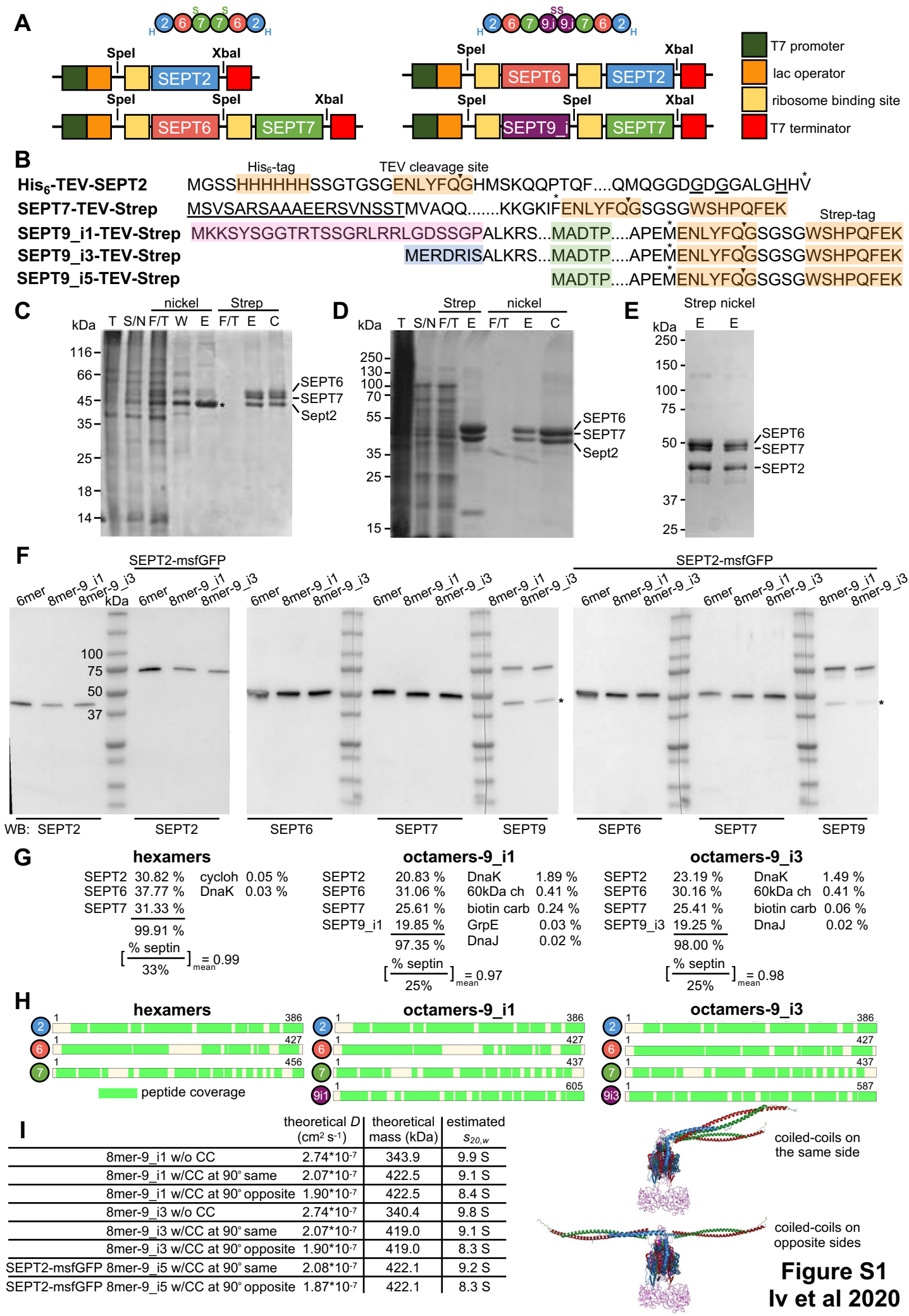
Figure S4. Modeling of the N-terminal extensions of SEPT9_i1, SEPT9_i3 and SEPT9_i5. **(A)** Models of the N-terminal extensions of SEPT9_i3 (left) and SEPT9_i5 (right) as random coils in the context of the respective octamers (only the central SEPT9 dimer is shown for simplicity); compare with Fig. 1H for SEPT9_i1. The N-terminal sequence specific to SEPT9_i3 is depicted in orange. **(B-C)** Model of a human octamer-9_i1 built as in Fig. 1H, now depicting the N-terminal extension of SEPT9_i1 in an extended conformation as predicted by RaptorX (see Materials and methods for details). An *en face* view (left) and a side view after a 90° rotation (right) are shown. Generated models containing this extended conformation were used to calculate the theoretical sedimentation coefficients for comparison with the experimentally obtained ones (table in C). Such models predict sedimentation coefficients that are much smaller than the experimentally measured ones, suggesting that the N-terminal extensions adopt a more compact/globular conformation in solution and in the absence of any interacting partners. **(D)** The extended conformation of the N-terminal extension (NTE) of SEPT9_i1 (dashed rectangle in B) is annotated as follows. The N-terminal stretch, in orange, depicts the sequence specific to SEPT9_i1; the remaining of the sequence, starting at Ala26 (A26), is common with the other long SEPT9 isoforms (SEPT9_i2 and SEPT9_i3). Residues Met165 (M165) and Met252 (M252) depict the onset of the short isoforms SEPT9_i4 and SEPT9_i5, respectively. The N-terminus of SEPT9_i4 until the onset of the region shared with SEPT9_i5 is shown in red; the N-terminus of SEPT9_i5 until the onset of the $\alpha 0$ helix is shown in blue. Residues Arg106 (R106) and Ser111 (S111), in green, are mutated in HNA. The helix comprising residues KRAEVLG, colored in lime, depicts a putative imperfect repeat identified in Verdier-Pinard et al., 2017. **(E-F)** Alternative models for the NTE of Sept9_i1 (E) and the NTE of Sept9_i3 (F) as predicted by RaptorX. The N-terminal sequence specific to SEPT9_i3 is depicted in orange; both Phyre2 (A) and RaptorX (F) model this sequence as a helix. The color code of the annotated residues is the same as for (D). The β -strands in cyan in the left model in (F) depict short β -strands predicted previously in Verdier-Pinard et al., 2017. The asterisks in D-F point to the sequence SKQVEN modeled as a helix. All models shown in D-F are hypothetical; potentially disordered NTEs of SEPT9 could conceivably become structured in the presence of interacting surfaces, for example, cell membranes, and binding partners. Interestingly, the predicted contact probability matrices of both SEPT9_i1 and SEPT9_i3 suggest that the first half of their N-terminal extension, which is unique to the long SEPT9 isoforms, forms potentially a structured domain (brackets in D-F), whereas the second half, which corresponds to the sequence shared with the short isoforms SEPT9_i4 and SEPT9_i5, is largely disordered.

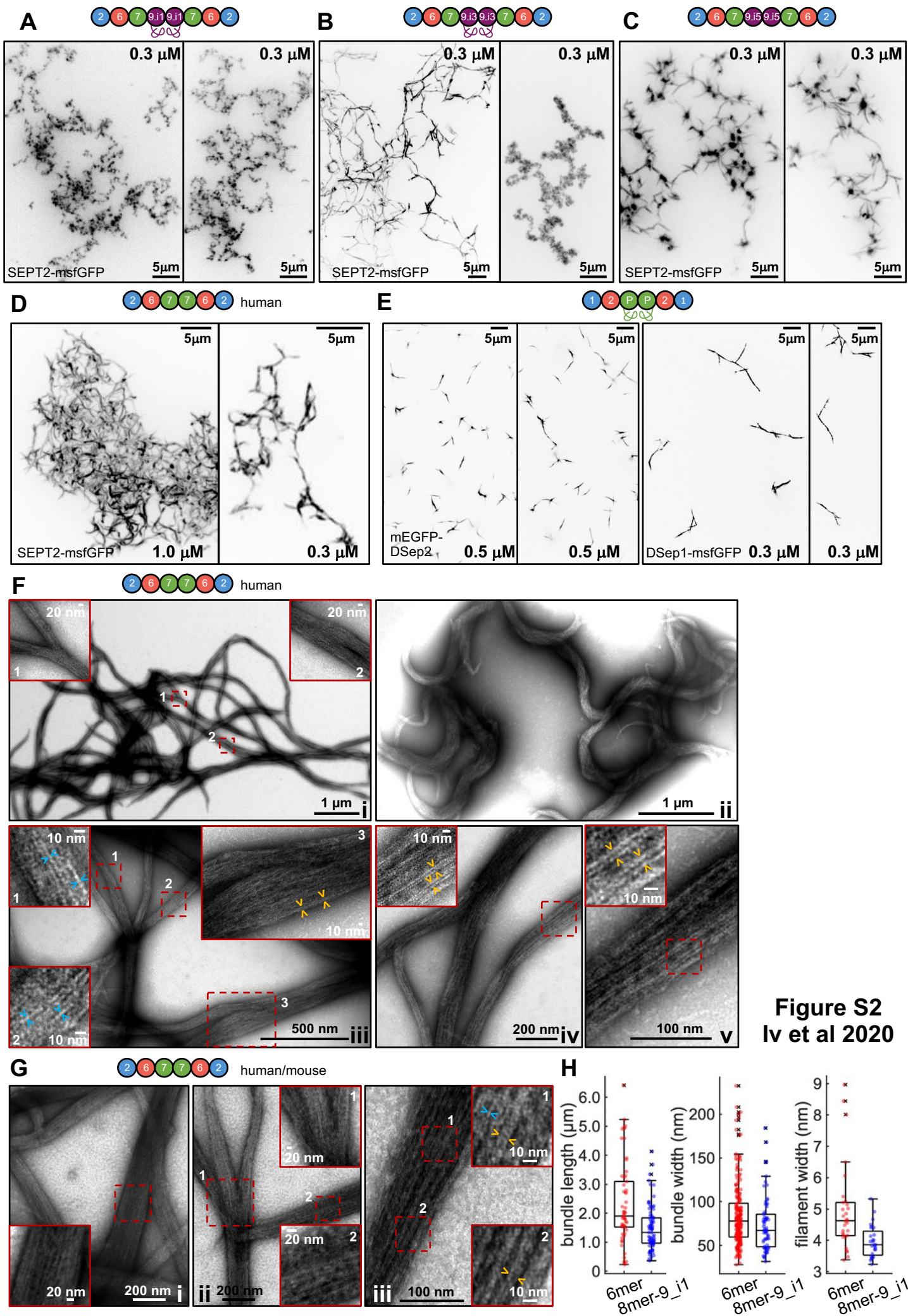
Table S1. Extinction coefficients and molecular masses used for concentration conversions for recombinant human septin complexes purified in this study.

Video 1. **Polymerization of recombinant human septin octamers-9_i3.** Optical sectioning (z-stack with a Δz interval of 0.5 μm) in the bulk of a flow channel depicting SEPT2-msfGFP human septin octamer-9_i3 polymerized at 0.3 μM (100% GFP-septins) by dilution into low-salt (50 mM KCl) buffer. Spinning disk fluorescence images displayed at 5 frames per second. Related to Fig. S2 B.

Video 2. **Reconstitution of single actin filaments.** Time-lapse sequence (Δt interval of 0.5 s) at the surface of a PLL-PEG passivated glass coverslip showing single fluctuating actin filaments at 1 μM . G-actin was polymerized in the presence of Alexa Fluor 568-phalloidin. Spinning disk fluorescence images displayed at 5 frames per second. A still image from this time lapse sequence is shown in Fig. 4A.

Video 3. **Actin filament cross-linking by recombinant human septin octamers-9_i1.** Time-lapse sequence (Δt interval of 0.5 s) at the surface of a PLL-PEG passivated glass coverslip showing cross-linked actin filaments (at 1 μM) in the presence of SEPT2-msfGFP human septin octamer-9_i1 at 0.3 μM (20% GFP-septins). G-actin was polymerized in the presence of Alexa Fluor 568-phalloidin. The actin channel is shown. Spinning disk fluorescence images, using an inverted grayscale, are displayed at 5 frames per second. A still image from this time lapse sequence is shown in Fig. 4C.





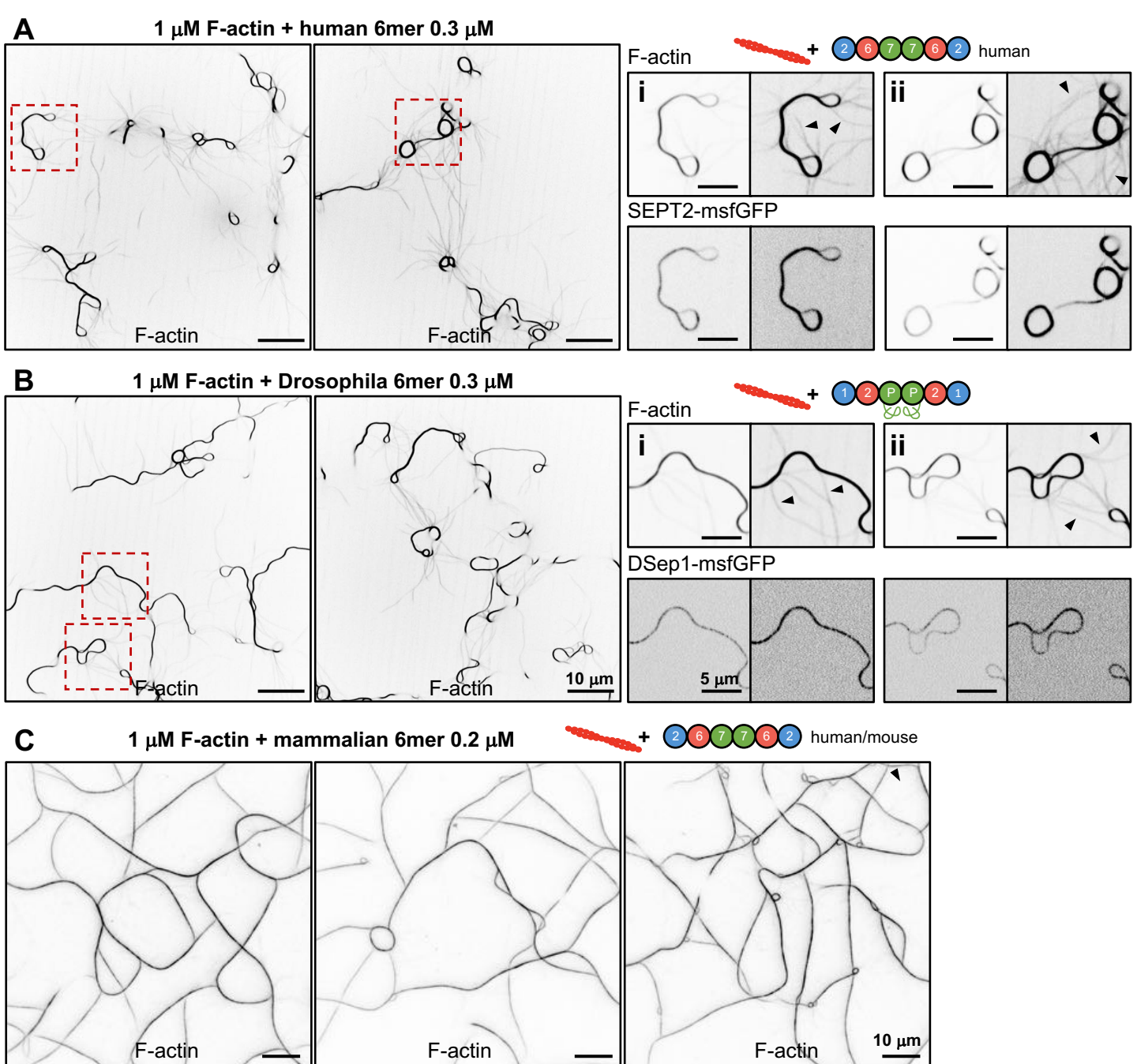


Figure S3
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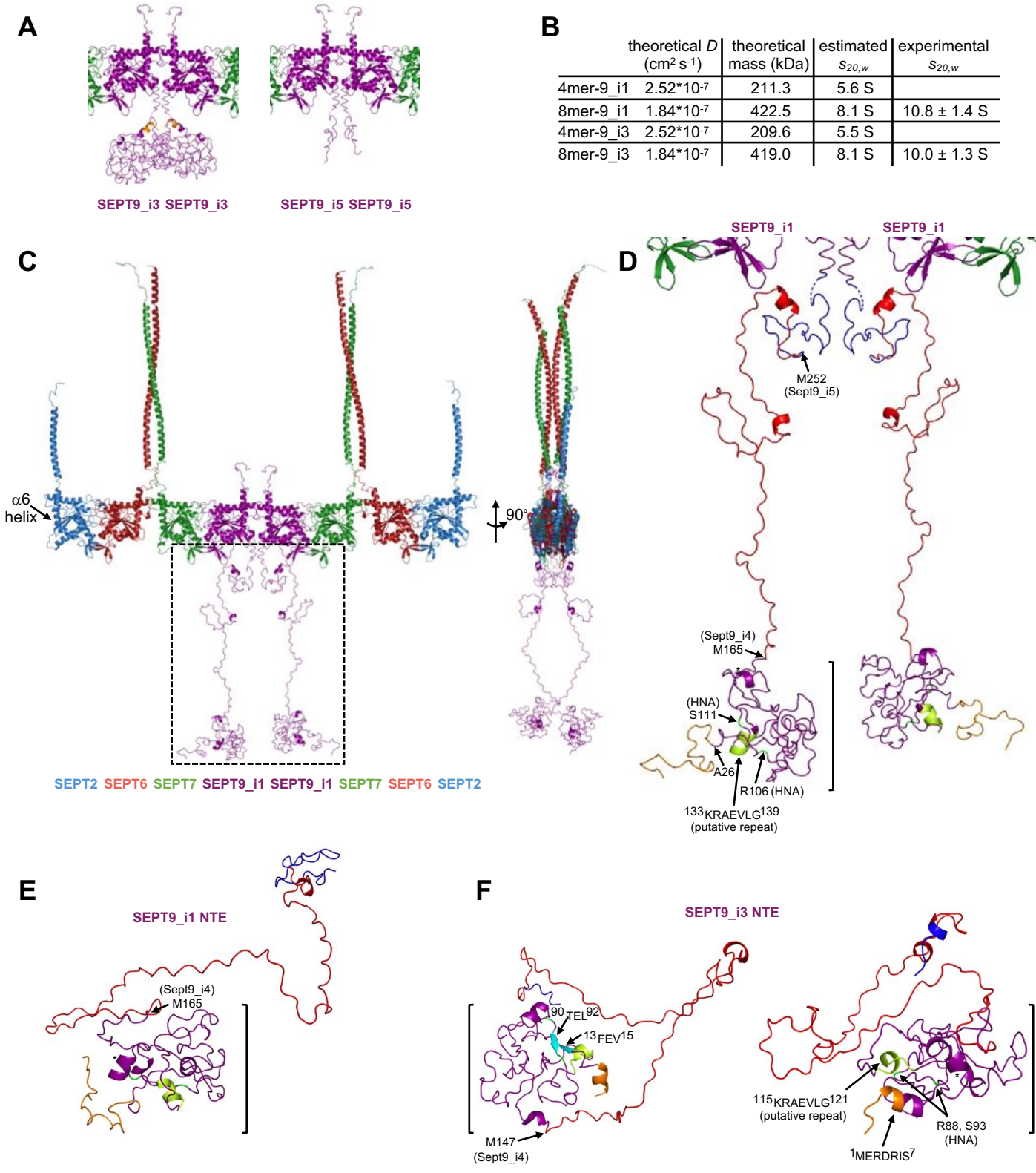


Figure S4
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	MW (kDa)	$\epsilon_{280\text{nm}}$ L.g ⁻¹ .cm ⁻¹	
human hexamers	291.8	0.563	1 g/L= 3.4 μM
human octamers-9_i1	422.5	0.505	1 g/L= 2.4 μM
human octamers-9_i3	419.0	0.502	1 g/L= 2.4 μM
human octamers-9_i5	368.7	0.570	1 g/L= 2.7 μM
SEPT2-msfGFP human hexamers	345.2	0.586	1 g/L= 2.9 μM
SEPT2-msfGFP human octamers-9_i1	475.9	0.527	1 g/L= 2.1 μM
SEPT2-msfGFP human octamers-9_i3	472.4	0.525	1 g/L= 2.1 μM
SEPT2-msfGFP human octamers-9_i5	422.1	0.588	1 g/L= 2.4 μM

Table S1. Extinction coefficients and molecular masses used for concentration conversions for recombinant human septin complexes purified in this study.