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Mira Kuzmić, Gerard Castro Linares, Jindřiška Leischner Fialová, Francois Iv, Danièle Salaün, et al.. Septin-microtubule association requires a MAP-like motif unique to Sept9 isoform 1 embedded into septin octamers. *Journal of Cell Science*, In press, 10.1101/2021.04.06.438596 . hal-03192486v1

HAL Id: hal-03192486

<https://hal.science/hal-03192486v1>

Submitted on 6 Oct 2021 (v1), last revised 2 Dec 2021 (v2)

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1 **Septin-microtubule association requires a MAP-like motif unique to Sept9 isoform 1**
2 **embedded into septin octamers**

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32 Keywords: actin/cytoskeleton/microtubule/septin/Sept9

47 **Abstract**

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Septins, a family of GTP-binding proteins assembling into higher order structures, interface with the membrane, actin filaments and microtubules, which positions them as important regulators of cytoarchitecture. Septin 9 (Sept9), which is frequently overexpressed in tumors and mutated in hereditary neuralgic amyotrophy (HNA), mediates the binding of septins to microtubules, but the molecular determinants of this interaction remained uncertain. We demonstrate that a short MAP-like motif unique to Sept9 isoform 1 (Sept9_i1) drives septin octamer-microtubule interaction in cells and *in vitro* reconstitutions. Septin-microtubule association requires polymerizable septin octamers harboring Sept9_i1. Although outside of the MAP-like motif, HNA mutations abrogates this association, identifying a putative regulatory domain. Removal of this domain from Sept9_i1 sequesters septins on microtubules, promotes microtubule stability and alters actomyosin fiber distribution and tension. Thus, we identify key molecular determinants and potential regulatory roles of septin-microtubule interaction, paving the way to deciphering the mechanisms underlying septin associated pathologies.

93 **Introduction**

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95 Microtubules and actin filaments are cytoskeletal polymers involved in key cellular
96 functions. Microtubules assemble from tubulin dimers to form hollow tubes that have the ability
97 to switch between growing and shortening phases, a process called dynamic instability.
98 Microtubule diversity arises, from tubulin isotypes, from post-translational modifications,
99 including acetylation and glutamylation, often associated with stable sub-populations of
100 microtubules, and from associations with specific microtubule-associated proteins (MAPs) that
101 modulate microtubule dynamics (Janke & Magiera, 2020). Microtubules are critically involved
102 in the segregation of chromosomes during cell division and the directed transport of various
103 intracellular cargoes (Carlton, Jones et al., 2020). Actin monomers assemble into double helical
104 strands that in cells are often cross-linked into bundles and networks (Maninova, Caslavsky et
105 al., 2017). In particular, the presence of associated myosins confers contractility to these actin
106 fiber assemblies, that is essential during both cell division and migration. Such bundled
107 actomyosin fibers form stress fibers that are qualified as peripheral and ventral when running
108 near the cortex and the cell base, respectively. Stress fibers connect focal adhesions and the
109 perinuclear actin cap linked to the top of the nucleus controlling its position and shape (Sneider,
110 Hah et al., 2019). Several biological processes depend on the coordinated regulation of the actin
111 and microtubule cytoskeletons, mediated by a diversity of molecular crosstalk between the two
112 cytoskeletons. Direct and indirect contacts are involved, for instances, in cytokinetic furrow
113 positioning, cell migration steering or maturation of neuronal dendritic spines (Dogterom &
114 Koenderink, 2019).

115 Septins are GTP-binding protein that form heteropolymeric complexes associating with
116 membranes, actin filaments and a subset of microtubules (Woods & Gladfelter, 2020). Like yeast
117 and *Drosophila* septins, human septin hexameric and octameric protomers can polymerize into
118 filaments (Bertin, McMurray et al., 2012, DeRose, Kelley et al., 2020, Iv, Martins et al., 2021,
119 Ong, Wloka et al., 2014, Soroor, Kim et al., 2021, Szuba, Bano et al., 2020, Valadares, d' Muniz
120 Pereira et al., 2017). Septins are thought to function as diffusion barriers for protein
121 compartmentalization or as scaffolds for protein-protein interactions during cells division and in
122 an increasing number of processes during interphase (Fung, Dai et al., 2014, Marquardt, Chen et
123 al., 2019, Mostowy & Cossart, 2012). During cytokinesis, septins are recruited to the sub-
124 membrane cortex by anillin (Oegema, Savoian et al., 2000, Renshaw, Liu et al., 2014) and co-
125 localize with the constricting actomyosin ring, prior to the specification and the maintenance of
126 the ingression furrow position by microtubules (Straight & Field, 2000, Verma & Maresca,
127 2019). Septins appear also to control abscission (Addi, Bai et al., 2018, Estey, Di Ciano-Oliveira
128 et al., 2010, Kim, Froese et al., 2011, Kinoshita, Kumar et al., 1997, Renshaw et al., 2014, Surka,
129 Tsang et al., 2002) that achieves cell division. It is still unclear how human septins interface with
130 microtubules and the acto-myosin networks. Septins form a collar structure at the neck of
131 dendritic spines, nanotubule-like protrusions and micro-tentacles to guide microtubules (Nolke,
132 Schwan et al., 2016, Ostevold, Melendez et al., 2017, Tada, Simonetta et al., 2007, Yadav, Osés-
133 Prieto et al., 2017). In adherent cells, septins concentrate on ventral stress fibers, and
134 occasionally on perinuclear microtubules, depending on the cell type. Despite studies showing
135 that septins regulate the traffic of motor proteins, like kinesin, the binding of MAPs and the
136 guiding of microtubules in polarizing epithelia (Spiliotis, 2010), the functional consequences of
137 septin-microtubule interaction are still largely unknown.

138 The 13 human septin genes encode four protein sequence homology-groups named after
139 Sept2, Sept6, Sept7 and Sept3; septins from each group interact with each other in a specific
140 order to form hexamers and octamers (Kim et al., 2011, Sellin, Stenmark et al., 2012, Sirajuddin,
141 Farkasovsky et al., 2007). Septin octamers differ from hexamers by the addition of septins from
142 the Sept3 group, with their arrangement recently determined as Sept2-Sept6-Sept7-Sept3-Sept3-
143 Sept7-Sept6-Sept2 (Mendonca, Macedo et al., 2019, Soroor et al., 2021). Septins include a
144 conserved GTP-binding domain (G domain) flanked by variable N- and C-termini. Septins
145 assemble by the G domain on one side forming the G:G interface and by the N- and C- termini
146 forming the NC:NC interface on the opposite side. This results in
147 (NCSept2_{G:G}Sept6_{NC:NC}Sept7_{G:G}Sept7_{NC:NC}Sept6_{G:G}Sept2_{NC}) hexamers and
148 (NCSept2_{G:G}Sept6_{NC:NC}Sept7_{G:G}Sept3_{NC:NC}Sept3_{G:G}Sept7_{NC:NC}Sept6_{G:G}Sept2_{NC}) octamers that can
149 polymerize via a universal Sept2_{NC:NC}Sept2 interface, that is labile in presence of high salt
150 concentrations. In the Sept3 group, Sept9 has been the most studied because it is ubiquitously
151 expressed, and often overexpressed in tumors (Connolly, Abdesselam et al., 2011). Interestingly
152 Sept9 is mutated in a large number of hereditary neuralgic amyotrophy (HNA) patients (Collie,
153 Landsverk et al., 2010, Hannibal, Ruzzo et al., 2009, Kuhlenbaumer, Hannibal et al., 2005).
154 Alternative splicing resulting in alternative translation start sites gives rise to five Sept9 isoforms
155 (Sept9_i1 to_i5) differing by the length and/or sequence of their N-terminus and by their
156 functions (Connolly, Yang et al., 2011, McIlhatton, Burrows et al., 2001, Verdier-Pinard, Salaun
157 et al., 2017). Sept9_i1, _i2 and _i3 long isoforms share a common structurally disordered N-
158 terminal domain (common N-ter) that was proposed, based on *in vitro* studies, to mediate
159 binding to microtubules (Bai, Bowen et al., 2013). However, several studies indicate that only
160 the isoform 1 of Sept9 (Sept9_i1) drives the association of septins with microtubules (Nagata,
161 Kawajiri et al., 2003, Sellin et al., 2012, Surka et al., 2002, Verdier-Pinard et al., 2017). In order
162 to understand the contribution of septin-microtubule interaction to cell physiology and disease, it
163 is critical to identify and characterize precisely the molecular determinants of this interaction.

164
165 In the present study, we show that septin association with microtubules requires a MAP-
166 like motif specific of Sept9_i1 and the integration of Sept9_i1 within polymerized septin
167 octamers. *In vitro* reconstitution allowed us to demonstrate a direct and specific interaction of
168 Sept9_i1-harboring septin octamers with microtubules, which slows down depolymerization of
169 microtubules. Based on this molecular characterization, we designed specific genetic variants,
170 including HNA-like mutations, which modulate Sept9_i1-microtubule interaction and we show
171 that relocalizing septins to microtubules disturbs stress fiber distribution and tension.

172 173 **Results**

174
175 **Different Sept9 isoform expression profiles are associated with distinct**
176 **octamers/hexamers ratios and cytoskeletal localization.** We have analyzed septin expression
177 profiles in commonly used cell models, namely U2OS osteosarcoma, HeLa cervical carcinoma
178 cells, RPE1 retinal epithelial cells and SKBr3 breast carcinoma cells (Fig. 1a). Sept7 being
179 unique in its group, it is essential for the formation of hexamers and octamers (Supplementary
180 Fig. 1d). The four cell lines examined expressed very similar levels of Sept7. In the Sept2 group,
181 Sept2 was expressed in all cell lines and Sept5 was only detected in U2OS cells. Expression of
182 septins from the Sept6 group was more variable, except for Sept8 that was expressed at similar
183 levels in all cell lines. U2OS and HeLa cells expressed similar levels of total Sept9. However,

184 U2OS cells expressed mostly isoform 3, whereas HeLa cells expressed mostly isoform 1. RPE1
185 and SKBr3 cells expressed higher levels of Sept9 with similar levels of Sept9_i1 and Sept9_i2,
186 and no or very low levels of Sept9_i3.

187 To assess how Sept9 expression profiles affected its incorporation in septin hexamers and
188 octamers, we extracted cells in conditions that preserved oligomers and performed native gel
189 electrophoresis and analysis by Western blotting (Fig. 1b and Supplementary Fig. 1). Based on
190 the detection of the essential Sept7 and pan-expressed Sept8, U2OS and HeLa cells contained
191 more hexamers than octamers, as opposed to RPE1 and SKBr3 cells. This result is consistent
192 with higher Sept9 expression levels in RPE1 and SKBr3 leading to higher levels of octamers. In
193 addition, our results indicate that long Sept9 isoforms incorporate indifferently in octamers. We
194 did not detect smaller septin assemblies containing Sept9 monomers or dimers, suggesting that,
195 at endogenous levels, the entire pool of Sept9 is stably incorporated into octamers.

196 Next, we examined the localization of Sept9 in these cell lines either in interphase or in
197 late cytokinesis (Fig. 1c, d). We observed that Sept9 was associated with actin fibers in all
198 interphase cells, especially on ventral stress fibers (Fig. 1c, d). Interestingly, consistent with
199 previous observation (Verdier-Pinard et al., 2017), Sept9 co-localized with a subpopulation of
200 acetylated microtubules in HeLa and SKBr3 cells; in cytokinetic cells, Sept9 concentrated
201 cortically at the ingression furrow and on the thick bundles of acetylated microtubules contained
202 in the intercellular bridge. In U2OS cells, which express primarily Sept9_i3, septins associated
203 with the actin cytoskeleton but did not co-localize with microtubules. These results indicate that
204 the association of septins with microtubules is dependent on the presence of octamers harboring
205 Sept9_i1. It is less clear why in RPE1 cells, despite Sept9_i1 levels comparable to SKBr3 cells,
206 there was no association of Sept9 with microtubules, except in primary cilia (Fig. 1e).

207
208 **Sept9_i1 interacts with microtubules via a specific MAP-like motif.** The fact that
209 only Sept9_i1, but not the closely related i2 and i3 isoforms, is required for septins to bind
210 microtubules identifies the sequence formed by the first specific 25 amino acid residues of the
211 Sept9_i1 protein, as specific to this isoform and as key for its interaction with microtubules
212 (Verdier-Pinard et al., 2017). This sequence being well conserved in vertebrates (Supplementary
213 Fig. 2), it is also indicative of an important function. Searching for similar sequences in proteins
214 related to the microtubule cytoskeleton by using the NCBI Blastp tool, we found the Arabidopsis
215 thaliana AIR9 protein, a plant MAP involved in cytokinesis (Buschmann, Chan et al., 2006). The
216 sequence of AIR9 similar to the Sept9_i1 specific sequence was located in the AIR9-microtubule
217 binding domain (AIR9 1-234) (Buschmann et al., 2006). Alignment of these two sequences
218 reveals a block of similar sequence that we named AIR-9-like (Fig. 2a). Another sequence,
219 located upstream in the AIR9 N-terminus (aa 46-78), presents a lower similarity with the
220 Sept9_i1 specific sequence (Fig. 2a). Strikingly, the AIR9-like block had similarities with
221 canonical repeats found in the microtubule binding domains (MBDs) of the structural human
222 MAPs, tau, MAP2 and MAP4, which are known to interact directly with the surface of
223 microtubules (Fig. 2a). An upstream smaller block in the Sept9_i1 specific sequence showed
224 similarities with the MAP4 R2 repeat. Thus, Sept9_i1 displays a putative MBD, similar to the
225 MBD repeats of several MAPs and constituted by a MAP4 R2-like sequence followed by an
226 AIR9-like sequence.

227 To unequivocally demonstrate that this motif is actually the MBD of Sept9_i1, we
228 examined the localization of Sept9_i1 mutants (C-terminally tagged with GFP), presenting
229 deletions or point mutations in the first 25 amino acid residues (Fig. 2b), in U2OS cells (Fig. 2c,

230 d and Supplementary Fig. 3a-c). U2OS cells were chosen because they express very low levels
231 of Sept9_i1 (Fig. 1a), and show no Sept9-microtubule co-localization (Fig. 1c, d). In addition,
232 U2OS cells were amenable to simultaneous siRNA-mediated knockdown (KD) of Sept9 and
233 expression of exogenous Sept9_i1-GFP constructs at near-endogenous Sept9 levels
234 (Supplementary Fig. 3a). Wild type Sept9_i1 co-localized with actin in all cells and with
235 microtubules in more than 60% of the cells. Sept9_i3 and the Δ 1-25 Sept9_i1 mutant also
236 associated with actin fibers, but never localized to microtubules (Fig. 2c, d, f and Supplementary
237 Fig. 3b, d). This result is consistent with our previous observations of Sept9_i3 and Sept9_i1 Δ 1-
238 25 vs Sept9_i1 localization in SKBr3 cells (using N-terminally tagged constructs) (Verdier-
239 Pinard et al., 2017). Deletion of the AIR9-like portion (Δ 10-25 mutant) or of the MAP4 R2-like
240 portion (Δ 1-7 mutant) totally or largely abrogated Sept9 binding to microtubules, respectively
241 (Fig. 2c and Supplementary Fig. 3b). Next, we introduced point mutations in the AIR9-like
242 portion (Fig. 2b). Independent mutations of two sets of contiguous serine residues (S12A/S13A
243 and S22A/S23A), conserved in the AIR9 MBD, did not significantly alter the co-localization of
244 Sept9 with microtubules (Fig. 2c, d and Supplementary Fig. 3b, c). Importantly, mutations of the
245 two arginine residues (R10A/R15A), conserved in all vertebrates (Supplementary Fig. 2) and
246 present in the AIR9 MBD, completely abrogated the binding of Sept9 to microtubules (Fig. 2c, d
247 and Supplementary Fig. 3b, c). Collectively, our data show that the Sept9_i1 specific N-terminal
248 sequence is an MBD analogous to the ones found in human MAPs and the plant AIR9 MAP. Of
249 note, in contrast to MAP MBDs, the Sept9_i1 specific sequence is not tandem-repeated and lacks
250 upstream conserved residues (Fig. 2a).

251 A previous report proposed, based on *in vitro* studies using recombinant polypeptides,
252 that Sept9_i1₆₁₋₁₁₃ within the N-terminal region common to all Sept9 long isoforms (i1, i2 and i3)
253 was the main contributor to microtubule binding (Bai et al., 2013). To evaluate a potential
254 contribution of Sept9 N-terminal region common to all long isoforms (common N-ter), we
255 generated a chimeric Sept9 deleted of the entire common N-ter by fusing the isoform 1 specific
256 sequence to Sept9_i5, the shortest Sept9 isoform, and named it Sept9_i1-i5 (Fig. 2e). Whereas
257 Sept9_i5 localized strictly to actin fibers (Fig. 2f, h and Supplementary Fig. 3e), its fusion to the
258 Sept9_i1 specific motif was sufficient to localize septins to microtubules in all interphase cells.
259 Surprisingly, this was accompanied by the complete loss of Sept9 co-localization with actin
260 fibers (Fig. 2f, h). Consistent with these findings, expression of Sept9_i1-i5 during cytokinesis
261 increased strongly the percentage of cells showing Sept9 association with microtubule bundles at
262 the intercellular bridge (Supplementary Fig. 3d).

263 Our results demonstrate that the Sept9_i1 MBD is required and sufficient to target Sept9
264 to microtubules, whereas the N-terminal region common to all long isoforms is dispensable.
265 Interestingly the Sept9_i1-i5 is much more efficient than Sept9_1 for targeting septins to
266 microtubules (Fig. 2f, h and Supplementary Fig. 3d), hinting that the common N-ter might have a
267 negative regulatory function. Sept9 is mutated in hereditary neuralgic amyotrophy (HNA)
268 patients. HNA-associated mutations are located in the Sept9 common N-ter (Kuhlenbaumer et
269 al., 2005), but their impact on Sept9 function remains to be elucidated. We introduced separately
270 the two most frequently described HNA mutations (R106W or S111F) in Sept9_i1 constructs
271 and evaluated their impact on Sept9_i1 localization. Both Sept9_i1 R106W and S111F
272 maintained co-localization with ventral stress fibers (Fig. 2g, h). However, both mutations
273 suppressed the association with microtubules (Fig. 2g, h). This result confirms that the common
274 N-ter, while not required for Sept9 microtubule binding per se, is an important regulatory domain
275 and provides novel insights into the molecular etiology of HNA.

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Sept9_i1 must be included in a polymerized septin octamer to associate with microtubules. In contrast to typical MBDs in structural MAPs, the Sept9_i1 specific sequence does not have tandem repetitions. We hypothesized that the dimerization of Sept9_i1 in octamers via the NC interface brings two Sept9_i1 specific sequences in close proximity, to form a MBD mimicking tandem arrangements of repeats. To test this hypothesis and the oligomeric environment required for microtubule binding, we introduced point mutations into Sept9_i1 that should disturb the NC interface to generate tetramers, or the G interface to generate non-incorporated Sept9_i1 (Sirajuddin et al., 2007) (Fig. 3a). We then expressed these constructs in U2OS cells in place of endogenous Sept9 (KD via siRNA). Importantly, re-expression of wild type Sept9_i1-GFP restored close to endogenous levels of octamers and hexamers (Fig. 3a). As expected, most of the Sept9_i1 G interface mutant (Sept9_i1 G_{mut}) did not incorporate in octamers (Fig. 3a). Instead, it migrated with an apparent molecular weight of 138 kDa, corresponding most likely to monomeric Sept9_i1, whose gel motility is retarded by the long and disordered N-terminus (Sellin et al., 2012) (Supplementary Fig. 1a). Based on previous studies (Bertin, McMurray et al., 2010, Kim et al., 2011, Sirajuddin et al., 2007), we generated two Sept9_i1 NC interface mutants with mutations in the $\alpha 0$ helix either in the basic residue stretch (Sept9_i1 NC_{mut1}) or upstream of this stretch in key hydrophobic residues (Sept9_i1 NC_{mut2}). Sept9_i1 NC_{mut1} incorporated in tetramers and Sept9_i1 NC_{mut2} incorporated partly in tetramers, but mostly migrated like the monomeric protein (Fig. 3a), indicating that mutations in Sept9_i1 NC_{mut2} might affect both NC and G interfaces. Importantly, we observed no association of Sept9_i1 G or NC mutants with microtubules, but rather a diffuse cytosolic distribution (Fig. 3b). These results show that neither septin tetramers nor Sept9_i1 monomers bind to microtubules, and that the octameric septin context, which brings together two Sept9 units via their NC interface, is mandatory for Sept9_i1 association with microtubules.

Next, we evaluated if septin octamers harboring Sept9_i1 bind microtubules by themselves or if they need to assemble into longer polymers via Sept2:Sept2 NC interfaces. Mutation of the Sept2 NC interface (Kim, Froese et al., 2012, Sirajuddin et al., 2007) (Sept2 NC_{mut}) should produce a mixture of isolated octamers and hexamers in cells (Fig. 3c). To evaluate its impact on Sept9_i1 localization, we knocked down both Sept9 and Sept2, and re-expressed Sept9_i1 (tagged with GFP to monitor Sept9 localization), together with wild type or Sept2 NC_{mut} (tagged with mApple to identify transfected cells). Using native gel separation, we verified that wild type Sept2 and Sept2 NC_{mut} generated the same relative expression levels of octamers and hexamers (Fig. 3c). However, when Sept2 NC_{mut} was expressed Sept9_i1 (and Sept2) no longer co-localized with microtubules (Fig. 3d). Thus, the association of Sept9_i1 with microtubules requires its incorporation in polymerizable octamers.

Sept9_i1 associates with stable microtubules. Because of the sequence similarities between Sept9_i1 and MAP4 MBDs, the two proteins might use the same tubulin interdimer binding pocket at the surface of microtubules (Kellogg, Hejab et al., 2018, Shigematsu, Imasaki et al., 2018). At endogenous Sept9_i1 levels in SKBr3 cells, we observed that MAP4 associated with the entire microtubule cytoskeleton, whereas Sept7 was concentrated on acetylated bundled microtubules (Fig. 4a). In U2OS cells transfected with Sept9_i1, the same differential distribution between MAP4 and Sept9_i1 was observed. When the Sept9_i1-i5 chimera, which showed a stronger affinity for microtubules, was expressed, MAP4 was absent on many

321 microtubule bundles (Fig. 4b). These observations suggest that the binding sites of Sept9_i1
322 MBD and MAP repeats partially overlap on the microtubule lattice.

323 As we repeatedly observed the preference of Sept9_i1 for acetylated microtubules (Fig.
324 1d, e, Fig. 2d, h, Fig. 3b, d, Fig. 4a and Supplementary Fig. 3c, g), we wondered whether
325 microtubule acetylation was a determinant of septin binding. We first examined whether the
326 unexpected lack of Sept9_i1 association with microtubules in RPE1 cells (Fig. 1c, d) was
327 associated with a deficit of microtubule acetylation. Tubulin acetylation levels in RPE1 cell line
328 were indeed low, especially compared to those in another retina epithelial cell line (ARPE19),
329 which expressed similar levels of Sept9_i1 (Fig. 5a) but showed frequent septin association with
330 microtubules (Fig. 5b, supplementary Fig. 3f). Other posttranslational modifications (PTMs) of
331 tubulins, such as polyglutamylation, that could affect Sept9_i1 binding (Froidevaux-Klipfel,
332 Targa et al., 2015, Spiliotis, Hunt et al., 2008) were equally low in these cell lines (Fig. 5a).
333 Moreover, treating RPE1 cells with paclitaxel, a drug that induces acetylation and bundling of
334 microtubules in cells, induced the association of septins with microtubules (Fig. 5c). Of note, the
335 paclitaxel-driven re-localization of septins to microtubules was dependent on the expression of
336 Sept9_i1 (Fig. 4c and Fig. 5c). Thus, Sept9_i1 association with microtubules appears to be
337 correlated with tubulin acetylation. To determine if paclitaxel-induced tubulin acetylation was
338 responsible for re-localization of septins on microtubules, α TAT1, the major tubulin acetyl
339 transferase (Janke & Montagnac, 2017), was knocked down to prevent tubulin acetylation, prior
340 to paclitaxel treatment (Fig. 5d). This experiment clearly showed that re-localization of septins
341 on paclitaxel-bundled microtubules was independent of tubulin acetylation levels (Fig. 5d).

342 Finally, we evaluated whether Sept9_i1, like MAPs, contributes to microtubule
343 stabilization in the presence of nocodazole, a microtubule-depolymerizing agent. We observed
344 that, whereas Sept9_i1 moderately stabilized microtubules, Sept9_i1-i5 strongly promoted the
345 resistance of microtubules against nocodazole-induced depolymerization (Fig. 4c, d and
346 Supplementary Fig. 3g). Of note, depolymerization of microtubules allowed Sept9_i1-i5 to re-
347 localize to actin fibers in nearly 60% of the cells (Fig. 4c).

348 Collectively, these data establish that septin octamers harboring Sept9_i1 associate with a
349 population of stable bundled microtubules in cells, independently of their acetylation levels, and
350 contribute to microtubule stabilization.

351

352 **Recombinant septin octamers harboring Sept9_i1 interact directly and specifically**
353 **with microtubules and slow down their depolymerization.** In order to assess if recombinant
354 octamers harboring (Oct_9i1) are able to bind directly to microtubules, we purified recombinant
355 septin octamers (Sept2-Sept6-Sept7-Sept9_i1-Sept9_i1-Sept7-Sept6-Sept2 and Sept2-Sept6-
356 Sept7-Sept9_i3-Sept9_i3-Sept7-Sept6-Sept2)(Iv et al., 2021) (Supplementary Fig. 1b) and
357 reconstituted dynamic microtubules made from purified tubulin α/β -heterodimers in the presence
358 of each type of octamer. Total internal reflection fluorescence (TIRF) microscopy allowed
359 visualizing both dynamic microtubules incorporating rhodamine-tagged tubulin and septin
360 oligomers incorporating GFP-tagged Sept2 (Fig. 6a). Microtubules were polymerized from a 10
361 μ M tubulin heterodimer solution and in the presence of 10 to 300 nM of octamers, i.e. at
362 concentrations comparable to the tubulin α/β -heterodimer (\sim 10 μ M) and septin complex (\sim 400
363 nM) concentrations measured in Hela cells (Hein, Hubner et al., 2015).

364 We clearly observed Oct_9i1 binding to microtubules at the lowest concentration (10 nM,
365 Fig. 6b, c). Oct_9i1 diffused randomly on the lattice of dynamic microtubules (Fig. 6c and
366 Supplementary Movie 1), and were also on the stable microtubule seeds. On kymographs,

367 diffusion on microtubules during the growing phases was clearly apparent, with a reduced
368 presence on the microtubule seeds (Fig. 6c). Of note, upon microtubule depolymerization,
369 Oct_9i1 accumulated and remained on seeds, then diffused again on the microtubule upon its
370 regrowth (Fig. 6c). At 10 nM concentration, there were no detectable recombinant octamers
371 harboring Sept9_i3 (Oct_9i3) on dynamic or stable seed microtubules. Decoration of
372 microtubules increased with increasing concentrations of Oct_9i1, until saturation at 300 nM
373 (Fig. 6b, c and Supplementary Movie 1). In comparison, Oct_9i3 only showed significant
374 binding at the highest concentration (300 nM), which was restricted to the stable microtubule
375 seeds (Fig. 6b, c and Supplementary Movie 2). Altogether these results indicate that octamers
376 harboring Sept9_i1 bind specifically and directly dynamic individual microtubules.

377 We then analyzed how the addition of Oct_9i1 or Oct_9i3, at 10 to 300 nM, affected
378 parameters of microtubule dynamic instability (Fig. 7a, b). Both types of octamer were similarly
379 and moderately affecting microtubule growth rate at intermediate concentrations. Strikingly,
380 Oct_9i1 induced a strong and dose-dependent decrease of the microtubule shortening rate, while
381 Oct_9i3 had no effect, even when used at the highest concentration. The catastrophe time
382 frequency was not consistently altered by the addition of either types of octamer. Intriguingly,
383 we observed that, solely in the presence of 200 nM or 300 nM of Oct_9i1, about 10% of
384 depolymerizing microtubules had an unusual curved tubulin-containing structure at their plus-
385 end that was decorated by septins (Fig. 7c and Supplementary Movie 3). These structures could
386 be made of few protofilaments whose shortening is slowed down by the association with
387 Oct_9i1.

388

389 **Re-localization of octamers from stress fibers to microtubules alters stress fiber** 390 **organization and function.**

391 Our observations indicate that the population Sept9_i1 octamers associate with both
392 microtubules and actin fibers and can shuttle between them. This was for instance illustrated by
393 the observation that the Sept9_i1-i5 chimera co-localized with microtubules only (Fig. 2f, h and
394 Fig. 4b, c), but was able to co-localize with actin fibers upon nocodazole-induced
395 depolymerization of microtubules (Fig. 4c). Thus, beyond its direct contribution to microtubule
396 stability and dynamics (this study) and microtubule-linked processes, e.g. cell resistance to
397 paclitaxel (Targa, Klipfel et al., 2019), the relative distribution of Sept9_i1 on microtubules vs
398 actin fibers might indirectly affect actomyosin fibers distribution and cellular events dependent
399 on their contractility. Previous studies showed that Sept9 associates with perinuclear actin fibers,
400 especially ventral stress fibers whose organization depends on Sept9 (Farrugia, Rodriguez et al.,
401 2020, Verdier-Pinard et al., 2017) (Supplementary Fig. 5a). We similarly observed that in U2OS
402 (Fig. 8a) and RPE1 cells (Supplementary Fig 5d), the knockdown of Sept9 strongly decreased
403 the percentage of cells with sub-nuclear stress fibers. To test the hypothesis that septin octamer
404 association with microtubules impacts actin fiber organization and function, we generated U2OS
405 cell lines stably expressing Sept9_i1 variants (Supplementary Fig. 4).

406 Expression of Sept9_i1-i5, only associating with microtubules, was concomitant with the
407 strong reduction in the percentage of cells having sub-nuclear stress fibers (Fig. 8b), while
408 expression of Sept9 constructs with no or reduced microtubule association (Sept9_i1 Δ 1-25,
409 Sept9_i1 R10A/R15A and Sept9_i3) preserved sub-nuclear stress fibers (Fig.8b). Likewise,
410 paclitaxel-induced re-localization of Sept9_i1 octamers from stress fibers to microtubule bundles
411 in RPE1 cells induced the loss of sub-nuclear stress fibers (Fig. 5c), whereas paclitaxel-treatment
412 of U2OS expressing Sept9_i3 as the major isoform did not result in any loss of sub-nuclear stress

413 fibers (Fig. 5c). Thus, the sequestration of septin octamers on microtubules is dependent on the
414 MBD of Sept9_i1 and induces a loss of sub-nuclear stress fibers. These results suggest that the
415 precise relative distribution of septins on microtubules vs on actin fibers is critical for an intact
416 actin cytoskeleton.

417 As a functional assay for testing the interplay between Sept9_i1-microtubule association
418 and stress fiber function, we plated U2OS cells KD for Sept9 or expressing different Sept9_i1
419 variants on adhesive micropatterns. Indeed, when plated on adhesive micropatterns, cells form
420 actin stress fibers across adhesives edges, and the convexity of the non-adhesive cell edges
421 provides a readout for stress fiber tension (They, Pepin et al., 2006). Partial or total localization
422 of septins on microtubules vs stress fibers in U2OS cells stably expressing Sept9_i1 or Sept9_i1-
423 i5, respectively, promoted the concavity of non-adhesive cell edges, reflective of the relaxation
424 of peripheral stress fibers compared to cells stably expressing Sept9_i1 Δ 1-25, that is solely
425 located on actin fibers (Fig. 8c). The knockdown of Sept9 in U2OS cells, leading to the loss of
426 septin octamers, induced a similar detectable relaxation of peripheral stress fiber (Fig. 8d).

427
428 Altogether, these results suggest that the relative abundance of octamers vs hexamers
429 and/or the relative proportion of septin octamers able to associate to microtubules contribute
430 critically to the maintenance of the actomyosin organization and tension. Our study indicates that
431 the propensity of octamers harboring Sept9_i1 to associate with microtubule depends on
432 Sept9_i1 expression levels, the state of its N-terminal regulatory domain, and the extent of
433 microtubule bundling.

434 435 **Discussion**

436
437 Septins associate in a dynamic way with different major constituents of the cell, i.e.
438 membranes, actin fibers and microtubules (Woods & Gladfelter, 2020). Septin octamers and
439 hexamers co-associate to form septin filaments (Bertin et al., 2012, Iv et al., 2021, Ong et al.,
440 2014, Soroor et al., 2021, Szuba et al., 2020, Valadares et al., 2017), whose properties may
441 depend in part on the relative abundance of incorporated octamers and hexamers. In addition, the
442 expression profile of different septin isoforms, including those of Sept9, may direct both
443 localization and function of septin octamers. It is thus critical to define precisely the molecular
444 basis of the different subcellular localizations of septin complexes, in order to develop tools
445 differentially altering their distribution. This will help us understand how the different septin
446 pools respectively contribute to cell physiology and pathology.

447 Earlier studies have shown that Sept9_1 has a preferential affinity for microtubules in
448 cells (Nagata et al., 2003, Sellin et al., 2012, Surka et al., 2002, Verdier-Pinard et al., 2017)
449 indicating that the first 25 aa residues defining the sequence unique to this long isoform was
450 responsible for specific binding to microtubules. In the present study, we identify a conserved
451 MAP-like MBD in Sept9_i1, including two distinct modules and key conserved residues whose
452 mutation abrogates microtubule binding. We and others have identified imperfect repeats
453 containing basic amino acid residues in the common long N-terminal sequence of Sept9_i1, _i2
454 and _i3 (Bai et al., 2013, Verdier-Pinard et al., 2017). These repeats were proposed to constitute
455 the MBD of Sept9 via electrostatic interactions with the acidic C-termini of β -tubulin exposed at
456 the surface of microtubules (Bai et al., 2013), in line with the accepted mode of interaction of
457 conventional MAPs at the time (Serrano, Montejo de Garcini et al., 1985). However, recent
458 advances in the determination of the structure of MAP tandem repeats bound to microtubules by

459 cryo-EM contradicted this model (Kellogg et al., 2018, Shigematsu et al., 2018). These studies
460 revealed that a sequence in Tau and MAP4 repeats anchors MAPs to a binding pocket at the
461 interface between consecutive tubulin heterodimers in protofilaments, independently of tubulin
462 acidic C-termini. We actually found numerous similarities between the MAPs repeated
463 sequences and the AIR9-like module of Sept9_i1 MBD, that are not present in the repeats shared
464 with the other Sept9 long isoforms. Most importantly, our data show that, contrary to the
465 Sept9_i1 MBD, the N-terminal region common to all long isoforms is not required for the
466 association of Sept9_i1 harboring octamers with microtubules in cells. Deletion of this region
467 actually favored septin association with microtubules at the expense of actin fibers. These
468 findings led us to speculate that the common N-terminal domain is not directly involved in the
469 interaction of Sept9_i1 with the microtubule lattice, but negatively regulates it. The imperfect
470 repeats of the common N-terminal domain were predicted to form short β -sheets whereas the rest
471 of the domain is disordered (Verdier-Pinard et al., 2017). One of the imperfect repeats of the
472 common N-terminal region is the locus of mutations detected in HNA patients (Collie et al.,
473 2010, Hannibal et al., 2009, Kuhlenbaumer et al., 2005). To this date the mechanisms whereby
474 Sept9 mutations contribute to this disease are not known. Remarkably, when we expressed
475 Sept9_i1 harboring HNA-like mutations, septin octamers were no longer able to associate with
476 microtubules, while still associating with actin fibers. This finding suggests that HNA-associated
477 mutations alter the putative “Sept9_i1 MBD regulatory region” providing clues for the
478 determination of the molecular mechanisms underlying HNA.

479 Importantly, we demonstrated that Sept9_i1 localizes septins on microtubules, only if it is
480 included in polymerizable septin octamers. At endogenous expression levels, in the diverse
481 cellular models we studied, we did not observe the presence of a significant pool of monomeric
482 Sept9 (Fig. 1b), which suggests that microtubule-associated Sept9 is wholly incorporated in
483 octamers in these cells. Conversely, the large excess of Sept9_i1 monomers in cells expressing
484 the Sept9_i1 G interface mutant was not associated with microtubules (Fig. 3a, b). Thus, *in vitro*
485 studies with isolated recombinant Sept9_i1 suggesting that Sept9_i1 can binds and bundle
486 microtubules by itself (Bai et al., 2013, Karasmanis, Phan et al., 2018, Nakos, Rosenberg et al.,
487 2019b) must be interpreted with caution.

488 In previous studies, recombinant Sept9_i3 or the common N-ter fragments were shown to
489 associate with microtubules, assembled from purified tubulin (Bai et al., 2013, Nakos et al.,
490 2019b). The discrepancy is most likely linked to the fact that in these *in vitro* experiments, Sept9
491 was used outside of its natural octameric context, often at high concentrations (up to 10-30 μ M
492 in pelleting assays) and using paclitaxel or GMPCPP stabilized microtubules in very low salt
493 buffers, which likely favored electrostatic interactions. Using recombinant octamers harboring
494 Sept9_i1 and Sept9_i3 (Iv et al., 2021), we observed that only those harboring Sept9_i1
495 associated with dynamics microtubules at low nanomolar concentrations, *in vitro*. Our *in vitro*
496 reconstitution experiments results are the first one to recapitulate the specific binding of Sept9_i1
497 octamers to microtubules observed in cells here and by others (Targa et al., 2019, Verdier-Pinard
498 et al., 2017). Of note, although recombinant Sept2-Sept6-Sept7-Sept7-Sept6-Sept2 hexamers
499 were found to interact with microtubules *in vitro* (Nakos, Radler et al., 2019a), this is not
500 consistent with the absence of co-localization of septin hexamers with microtubules, when Sept9
501 was knocked down in cells (Verdier-Pinard et al., 2017) (Fig 8a and Supplementary Fig. 1d).

502 Septins associate with a sub-population of bundled microtubules (Bai et al., 2013,
503 Bowen, Hwang et al., 2011, Nagata et al., 2003). We confirm that Sept9_i1 co-localize with
504 acetylated, bundled microtubules (much more selectively than structural MAPs, such as MAP4).

505 As we also show that microtubule acetylation is not a requirement, Sept9_i1 binding appears
506 rather to rely on microtubule bundling in cells. Intriguingly, microtubule bundling is not required
507 *in vitro*. This paradox will require other studies, including structural ones, to further characterize
508 the molecular bases of the association of septins with individual and bundled microtubules.

509 Our data show that octamers harboring Sept9_i1 slowed down microtubule
510 depolymerization *in vitro* and stabilized microtubules in cells treated with nocodazole. It will be
511 important to determine how these molecular events contribute to microtubule-dependent cellular
512 processes such as spatiotemporal sorting of vesicles and organelles (Karasmanis et al., 2018,
513 Spiliotis et al., 2008), formation of protrusive structures (Ghossoub, Hu et al., 2013, Hu, Bai et
514 al., 2012, Nolke et al., 2016, Ostevold et al., 2017, Tada et al., 2007, Yadav et al., 2017) or cell
515 survival against cytotoxic doses of paclitaxel (Targa et al., 2019).

516 Association of septins with microtubules is likely to be regulated by cell- and tissue-
517 specific contexts. Indeed, septin association with microtubules is clearly correlated to the specific
518 expression of the Sept9_i1 isoform and is sensitive to the bundling state of microtubules. Septin-
519 microtubule interaction might also be modulated by the presence of competing MAPs or PTMs
520 targeting the Sept9_i1 MBD and/or its putative regulatory domain. Importantly, we show that
521 deficient Sept9_i1 binding to microtubules is associated with a diseased state, as two distinct
522 HNA-like point mutations prevented Sept9 binding to microtubules. We are still far from
523 understanding the underlying mechanism leading to HNA, which might be related to
524 dysregulated microtubule function, but might be related also to an excessive association of
525 septins with actin fibers and perturbation of actomyosin functions. Thus, our work not only
526 establishes a new molecular basis for studying the physiological relevance of the association of
527 septin with microtubules, but also open new avenues in the difficult search for the causative
528 events leading to this rare neuropathy.

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551 **Materials and methods**

552

553 **Human Cell lines.**

554 U2OS (ATCC), SKBR-3 (ATCC), HeLa (a kind gift from Dr Patrice Dubreuil, CRCM,
555 Marseille, France), RPE-1 and ARPE19 (kind gifts from Dr Michael Sebbagh, CRCM,
556 Marseille) cells were maintained in Dulbecco's modified Eagle's medium (DMEM) (Gibco)
557 supplemented with 4 mM GlutaMAX™, 10 % fetal bovine serum (FBS) and 1% sodium
558 pyruvate (supplemented DMEM), except ARPE19 cells that were cultivated in DMEM/F12 (1:1
559 mixture of DMEM and Ham's F12, Gibco) supplemented as for DMEM. All cell lines were
560 maintained in the presence of 5% CO₂ humidified atmosphere at 37°C. RPE-1 cells were also
561 grown on coverslips coated with collagen (Corning, Cat#354236) until they reached confluency
562 and ciliogenesis was induced by growing cells in DMEM containing only 0.5% of fetal bovine
563 serum for 24 hours.

564

565 **Plasmids coding for fluorescent septin constructs and transfection**

566 To drive expression of the constructs in mammalian cells, we used the immediate early enhancer
567 and promoter of human cytomegalovirus (CMV promoter, 508 base pairs). Human Sept9_i1
568 cDNA was a gift from C. Montagna (Albert Einstein College of Medicine, USA). Human
569 Sept9_i3 cDNA was a gift from W. Trimble (University of Toronto, Canada). A synthetic human
570 Sept2 coding sequence (Eurofins Genomics, Germany) was generated using the codon usage of
571 mouse Sept2 except for the five codons that differ between the two species, for which we used
572 codons encoding the human residues. C-terminal green and red fluorescent protein fusions were
573 generated using monomeric (V206K) superfolder GFP (msfGFP) (Costantini, Fossati et al.,
574 2012, Cranfill, Sell et al., 2016, Pedelacq, Cabantous et al., 2006, Zacharias, Violin et al., 2002)
575 and monomeric Apple (mApple) (Cranfill et al., 2016, Shaner, Lin et al., 2008), respectively. All
576 constructs were generated with two-insert seamless cloning (In-Fusion HD Cloning Plus Kit
577 from Takara Bio, Cat# 638910) using NheI/BamHI linearized plasmid backbones (Addgene
578 plasmid #54759) and the oligonucleotide primer sequences (listed in Supplementary Table 1).
579 Primers for seamless cloning were Cloning Oligo (<60 bp) or EXTREmer (>60 bp) synthesis and
580 purification quality from Eurofins Genomics, Germany. Restriction enzymes were FastDigest
581 enzymes from Thermo Scientific. All plasmids were verified by sequencing (Eurofins Genomics,
582 Germany) after each cloning step, including the midpreps used for plasmid production.
583 Constructs were transiently transfected by nucleofection in U2OS cells (Amaxa nucleofector
584 program U2OS X-001, Lonza) as described previously (Verdier-Pinard et al., 2017).

585

586 **siRNAs and transfection**

587 The following 19-mer duplex siRNAs were purchased from LifeTechnologies: siRNA control
588 (siCtrl) targeting the E.Coli β galactosidase (LacZ) (5'-GCGGCUGCCGGAAUUUACC-3'),
589 Sept9 (5'-GGAUCUGAUUGAGGAUAAA-3') targeting the 3'UTR of all human Sept9 mRNA
590 variants, Sept7 (5'-CGACUACAUUGAUAGUAAA-3') targeting the human mRNA coding
591 region, α TAT1 (5'-CGCACCAACUGGCAAUUGA-3'), whose design was based on validated
592 siRNA α TAT1#2 from Shida et al (Shida, Cueva et al., 2010). Transfection of siRNA was
593 performed as described previously (Verdier-Pinard et al., 2017) either with Lipofectamine
594 RNAiMAX (Invitrogen, Cat# 13778075) or Amaxa cell line nucleofection kit V (Lonza,
595 Cat#VCA-1003).

596

597 **Drug treatments**

598 For some experiments, cells were treated for two hours with 2 μ M paclitaxel (Sigma-Aldrich,
599 Cat#T7191) or 10 μ M nocodazole (Sigma-Aldrich, Cat#M1404) in DMSO or with DMSO alone
600 (Sigma-Aldrich, Cat#D8418).

601

602 **Antibodies**

603 Labeling of septins on Western blots (WB) and/or on immunocytochemistry coverslip (ICC) was
604 achieved with the following antibodies: rabbit polyclonals against human Sept2 (Sigma-Aldrich,
605 Cat#HPA018481, WB), against human Sept9 (Sigma-Aldrich, Cat#HPA042564 and
606 HPA050627, WB and ICC; Proteintech, Cat#10769-1-AP, WB), against Septin 7 (IBL,
607 cat#18991, WB and ICC), against Sept11 (Sigma-Aldrich, Cat#SAB2102111, WB), against
608 human Sept5, against Sept8 (kind gifts from Barbara Zieger, University of Freiburg, Germany,
609 WB), against Sept6 (a kind gift from Makoto Kinoshita (Nagoya University, Japan, WB), against
610 Sept10 (Sigma, Cat#HPA047860, WB); rat monoclonals against human Sept9_i1 (clone 4D2A5,
611 WB) or human Sept9_i3 (clone 1A6C2, WB), and against human Sept7 (clone 10A7, WB and
612 ICC) were produced as described previously (Verdier-Pinard et al., 2017) (WB). Other
613 antibodies were against α -tubulin: mouse monoclonal (Sigma-Aldrich, DM1A Cat#05-829, WB
614 and ICC), rat monoclonal (Invitrogen, YL1/2 Cat#MA1-80017, ICC), mouse monoclonal anti-
615 acetylated K40 (Santa Cruz Biotechnology, 6-11B1 Cat#sc-23950, WB and ICC), against
616 tubulin: mouse monoclonal anti-glutamylated tubulin (Adipogen, GT-335 Cat#AG-20B-0020-
617 C100 and polyE Cat#AG-25B-0030-C050, WB). Labeling of actin was performed with
618 phalloidin-Atto 390 (Sigma-Aldrich, Cat#50556, ICC) or phalloidin-TRITC (Sigma-Aldrich,
619 Cat#P1951, ICC). Secondary antibodies conjugated with HRP were used for WB (Dako
620 Agilent). For ICC application, and for WB application when indicated in figures, the following
621 fluorophores conjugated to secondary antibodies were used: DYLIGHT405, AlexaFluor405,
622 AlexaFluor488 and AlexaFluor594 (Jackson, Immunoresearch) and AlexaFluor 647 (Invitrogen).

623

624 **U2OS cell lines stably co-expressing mCherry-H2B and Sept9-GFP constructs**

625 U2OS and HeLa cell lines stably expressing mCherry-H2B and a Sept9_X-GFP construct were
626 generated by co-transfecting the plasmid pBabeD hygro mCherry-Histone H2B (a kind gift from
627 Christophe Lachaud, CRCM, Marseille, France) with hygromycin B resistance and one of the
628 plasmids encoding for different Sept9 isoforms and mutants-GFP constructs, with geneticin
629 resistance. U2OS cells were nucleo-transfected (Amaxa nucleofector program U2OS X-001,
630 Lonza) with Amaxa kit V. Three days after transfection, co-expressing cells were selected with
631 hygromycin B (Invitrogen, Cat#10687-010) and geneticin (Gibco, Cat#10131-027) at 0.5 mg/mL
632 each. GFP and mCherry fluorescence positive cells were sorted by flow cytometry. Sorted cell
633 populations were cultured in supplemented DMEM in the presence of 0.5 mg/mL of antibiotics.
634 After three passages, cell line stocks were stored in liquid nitrogen.

635

636 **Micropatterning of U2OS cells**

637 6×10^3 U2OS cells KD for Sept7 or Sept9 or U2OS cells stably co-expressing mCherry-H2B and
638 Sept9-GFP constructs were seeded in each well formed by the assembly of a 35 mm collagen
639 coated coverslip with H-shaped medium size adhesive micropatterns (Cytoo, Cat#10-008-00-18)
640 and a magnetic four-well chamber (Cytoo, Cat#30-011) following manufacturer instructions.
641 Coverslips were fixed with 4% formaldehyde and processed for ICC as previously described

642 (Verdier-Pinard et al., 2017). Distance measurements were performed using the Graphics line
643 tool in Zen blue software as described in more details in Fig. 8c legend.

644 **SDS-PAGE and Western blotting**

645 Total protein extraction from cells in culture by scraping the cells off in NP-40 lysis buffer,
646 separation of proteins by SDS-PAGE using NuPAGE 4-12 % gradient Bis-Tris gels (Invitrogen,
647 Cat#NP0322BOX) and MOPS SDS running buffer (Invitrogen, Cat#NP0001-02) and Western
648 blotting of separated proteins on nitrocellulose were performed as described previously (Verdier-
649 Pinard et al., 2017).

650

651 **Separation of septin complexes by native gel electrophoresis and detection**

652 Native cellular protein extraction was carried out as published by Sellin et al. (Sellin, Stenmark
653 et al., 2014). Briefly, proteins from cells at 80% confluence in one 100 mm petri dish were
654 extracted in 40 μ l of native lysis buffer (80 mM PIPES pH 6.9, 2 mM $MgCl_2$, 4 mM EGTA,
655 0.2% saponin and protease inhibitor cocktail (Roche, Cat#04693159001); the extract was
656 incubated for 10 min on ice and centrifuged and the supernatant was supplemented with 0.45 M
657 sodium chloride, incubated on ice, centrifuged, concentrated and exchanged twice with
658 phosphate buffer (pH 7.5, 0.45 M NaCl, 1 mM EGTA and protease inhibitors) on an Amicon 30
659 kDa cut off concentrator (Merck, Cat# UFC 503096). Protein concentration was determined
660 using a BCA protein assay (Thermo Scientific, Cat#23223 and 23224), glycerol was added at 1:1
661 (vol:vol), and extracts stored at $-20^\circ C$ until use. Native PAGE was performed on 4-16%
662 NativePAGE Novex Bis-Tris polyacrylamide gels (Thermo Scientific, Cat#BN1002BOX)
663 following instructions from the manufacturer. Briefly, native protein extracts were mixed with 4x
664 sample buffer (Thermo Scientific, Cat#BN2003) and water (final sample buffer 1x, and about
665 150 mM NaCl). Eight μ g of proteins were loaded per lane. Unstained native protein molecular
666 weight standards were used (Thermo Scientific, Cat#LC0725) and stained with Coomassie blue
667 during electrophoresis. Protein complexes were transferred overnight on a PVDF membrane
668 under 20 V constant voltage. The membrane was partially destained with 25% MeOH and 10%
669 acetic acid, positions of native protein molecular weight standards were marked, and the
670 membrane was placed briefly in MeOH for complete destaining. The membrane was processed
671 for Western blot detection following the protocol used for nitrocellulose membranes described
672 previously (Verdier-Pinard et al., 2017).

673

674 **Immunocytochemistry**

675 Multiplex immunocytochemistry was carried out on cells cultured on collagen coated coverslips
676 followed by 4% formaldehyde fixation as described previously (Verdier-Pinard et al., 2017).
677 Images were acquired on a Zeiss structured light ApoTome microscope equipped with a 63x/1.4
678 Plan Apochromat objective and an AxioCam MRc5 camera using AxioVision software or a Zeiss
679 LSM880 META confocal microscope equipped with a 63x/1.46 Plan Apochromat objective and
680 a GaAsP detector using Zen software.

681

682 **Co-localization of septins with microtubules and with stress fibers.** Individual cells were
683 examined by ICC for co-localization of septins with total α -tubulin, acetylated K40 α -tubulin and
684 F-actin, from top to bottom. Cells were counted as positive for co-localization of septins with
685 microtubules or with F-actin when septin fluorescence signal was co-aligned with microtubules
686 and/or acetylated microtubules or with ventral stress fibers, respectively. The percentage of cells

687 positive for both septin-microtubule and septin-stress fibers co-localization, only positive for
688 septin-stress fibers co-localization and only positive for septin-microtubule co-localization were
689 designated in graphs as Microtubule+Actin, Actin, and Microtubule, respectively. The extent of
690 the co-localizations in each cell was not considered.

691

692 **Quantification of acetylated microtubules in cells**

693 The fluorescence surface corresponding to acetylated microtubules in individual cells was
694 determined using the ImageJ 1.48v software (Schneider, Rasband et al., 2012): the fluorescence
695 image of acetylated microtubules was converted in 8-bits, adjusted manually with the threshold
696 function for black and white levels to maximally remove black pixels unrelated to acetylated
697 microtubules, the actin image was synchronized with the acetylated microtubule image
698 (Synchronize Windows tool) and used to delimit the ROI corresponding to the entire cell surface
699 with the freehand selection tool, and finally the image was analyse using the measure function to
700 obtain the percentage of surface area represented by the remaining black pixels corresponding to
701 acetylated microtubule.

702

703 **Production and purification of recombinant human septin complexes**

704 Human septin hexameric and octameric complexes with either Sept9_i1 or Sept9_i3 were
705 produced, purified and analyzed as detailed in Iv et al (Iv et al., 2021). No difference in apparent
706 MW was observed between recombinant septin octamers or hexamers and those extracted from
707 cells after native gel separation and WB (Supplementary Fig. 1b, c).

708

709 **Septin/microtubule reconstitution and TIRF imaging**

710 GMPCPP-stabilized microtubule seeds serving as a nucleation site for dynamic microtubules
711 were prepared using an established double-cycle method (Gell, Bormuth et al., 2010). Briefly, a
712 ~22 μM mixture of tubulin (~11 μM tubulin dimers) in MRB80 (80 mM PIPES pH 6.8, 4 mM
713 MgCl_2 , 1 mM EGTA), composed of 75% unmodified tubulin dimers (Cytoskeleton, Inc.,
714 Ct#T240), 15% rhodamine-labeled tubulin dimers (Cytoskeleton, Inc., Cat#TL590M) and 10%
715 biotin-modified tubulin dimers (Cytoskeleton, Inc., Cat#T333P), was spun down using an
716 Airfuge® Air-driven ultracentrifuge (Beckman Coulter Inc., Brea, California, USA) for 5
717 minutes at 30 psi with a cold rotor. Then, the mixture was complemented with 1 mM GMPCPP
718 from a 10 mM stock solution, thus diluting the tubulin to 20 μM . This mixture was incubated at
719 37°C for 30 minutes to polymerize tubulin, and immediately airfused for 5 minutes at 30 psi
720 with the rotor at room temperature to pellet GMPCPP-stabilized microtubules. Afterwards, the
721 supernatant was discarded and the pellet was resuspended in warm MRB80 to a final tubulin
722 concentration of 20 μM , considering an 80% recovery of the tubulin. The mixture was incubated
723 on ice for 20 minutes to depolymerize the microtubules. Subsequently, it was complemented
724 with 1 mM GMPCPP, incubated at 37°C for 30 minutes to repolymerize the microtubules, and
725 airfused for 5 minutes at 30 psi with a warm rotor. The pellet, containing GMPCPP-stabilized
726 microtubule seeds, was resuspended in warm MRB80 supplemented with 10% glycerol, snap-
727 frozen, and kept at -80°C until use.

728 Nr. 1 Menzel coverslips (Thermo Fisher scientific, product number 11961988) and glass slides
729 (Thermo Fisher scientific, product number 11879022) were cleaned in base piranha solution (5%
730 hydrogen peroxide, 5% ammonium hydroxide) at 70°C for 10 minutes, washed extensively by
731 rinsing with Milli-Q water and stored in Milli-Q water for up to 5 days. Just before use, a
732 coverslip and a glass slide were blow dried with a stream of N_2 gas. Flow channels were

733 prepared by placing parallel 2x20 mm parafilm strips spaced by 2-3 mm between the glass slide
734 and the coverslip. The parafilm was melted by placing the chambers on a hotplate at 120°C.
735 After cooling down, the chambers were passivated by incubation with 0.2 mg/mL Poly(L-
736 lysine)-graft-biotinylated PEG (SuSoS, product number CHF560.00) in MRB80, 0.2 mg/mL
737 neutravidin in MRB80, 0.5mg/mL κ -casein in MRB80, and 1 wt% Pluronic F-127 in MRB80, in
738 that order and without intervening washing steps. The channels were washed with three channel
739 volumes (~30 μ L) of MRB80 at the end of the passivation process. Then the channels were
740 incubated for 10 minutes with an aliquot of GMPCPP-stabilized seeds that was quickly thawed at
741 37°C, allowing the microtubule seeds to bind to the surface via biotin-neutravidin interactions.
742 Microtubule polymerization was induced by immediately flushing into the channel 20 μ M
743 tubulin (3% rhodamine-labeled tubulin) in a 5:1 volume ratio of MRB80:septin buffer
744 complemented with 0.5 mg/mL κ -casein to prevent unspecific interactions, 0.1% methylcellulose
745 as a crowding agent, 1 mM ATP, 1 mM GTP and an oxygen scavenging system composed of 50
746 mM glucose, 200 μ g/ml catalase, 400 μ g/ml glucose-oxidase, and 4 mM DTT. A mixture of 90%
747 unlabelled and 10% msGFP-labeled septin hetero-hexamers or hetero-octamers (containing
748 either SEPT9_i1 or Sept9_i3) were added to the previous mix at different concentrations in the
749 range of 10 to 300 nM.

750 The samples were immediately imaged using a Nikon Ti2-E microscope complemented with a
751 Gataca iLAS2 azimuthal TIRF illumination system heated to 30°C using an Okolab incubator
752 system. The sample was illuminated with 488-nm and 561-nm lasers (Gataca laser combiner
753 iLAS2) to visualize the septin and the tubulin signals, respectively. To achieve fast imaging, the
754 fluorescence signal was split with a Cairn Research Optosplit II ByPass containing a Chroma ZT
755 543 rdc dichroic mirror and filtered with either a 525/50 or a 600/50 chroma bandpass filter. The
756 images were recorded with a Andor iXon Ultra 897 EM-CCD camera using an exposure time of
757 75 ms, for 10-20 minutes with a frame rate of 1 frame/second.

758 Microtubule dynamics were analysed by kymograph analysis (Bieling, Laan et al., 2007).
759 Kymographs were built with the reslice tool in FIJI (Schneider et al., 2012) on a manually drawn
760 line that went from the beginning of the microtubule seed to the tip of the microtubule in its
761 maximum length. In the kymographs, we can observe the positions of the microtubule tips over
762 time. The analysis was only done on the plus ends of the microtubules, which can be
763 distinguished from the minus ends via the longer final length and higher growth velocity of the
764 former. Growth and shortening rates were obtained as the slopes of manually fitted straight lines
765 on the growing or shortening contour phases, determined by manual inspection, of the
766 microtubule plus ends. The catastrophe rate was calculated as the inverse of the time that a
767 microtubule spends growing.

768

769 **Statistical analysis**

770 Unless indicated otherwise, statistical analysis was performed using GraphPad Prism software
771 and unpaired two-tailed t-test with Welch correction for cell related data or using the function
772 compare_means from the package ggpubr from R and two-tailed t-test with Benjamini &
773 Hochberg p-value correction (<https://rpkg.datanovia.com/ggpubr/index.html>) for the *in vitro*
774 data related to microtubule dynamics.

775

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1036 **Acknowledgements**

1037 This research received funding from the Agence Nationale pour la Recherche (ANR grant ANR-
1038 17-CE13-0014; SEPTIMORF) (to M.M. and P. V-P.). We thank Jeffrey den Haan (TU Delft,
1039 The Netherlands) for help with protein purification. This work was further financially supported
1040 by the Netherlands Organization for Scientific Research (NWO/OCW) through the 'BaSyC-
1041 Building a Synthetic Cell' Gravitation grant (024.003.019) (to G.K.).
1042

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1050 supervision, writing – original draft, writing – review & editing.
1051
1052
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1054 **Figure legends**

1055

1056 **Fig 1. Sept9 expression, incorporation in octamers and localization in human cell lines. a**
1057 Western blots of total protein extracts from U2OS, HeLa, RPE1 and SKBr3 cells probed with
1058 antibodies against septins and tubulin. **b** Native extracts from the same cell lines were analyzed
1059 by Western blotting for their content in both septin hexamers and octamers using antibodies
1060 against constitutively required Sept7 and ubiquitously expressed Sept8; septin octamers were
1061 also specifically labeled with a pan Sept9 antibody. Relative content in septin octamers and
1062 hexamers in each cell line based on three independent Sept7 WB is presented below the blots
1063 (mean \pm SEM). **c** and **d** Cellular localization by immunocytochemistry of Sept9 in the same cell
1064 lines, during interphase and cytokinesis; in **c**, the percentages of cells displaying both co-
1065 localization of Sept9 with microtubules and co-localization with stress fibers (Microtubule +
1066 Actin) or only stress fibers (Actin) were determined from three independent experiments based
1067 on a total of 90 cells (30 cells per experiment). Unpaired, two-tailed t-test with Welch's
1068 correction, ** $p < 0.01$, *** $p < 0.0005$, Microtubule and Actin localization vs in SKBr3; in **d**,
1069 white scale bars correspond to 5 μm , insets are two-fold zoomed regions framed by a white
1070 square in the corresponding original images. **e** RPE1 cells were starved for 24 h and
1071 immunofluorescence was performed as in **c** and **d**; white scale bars correspond to 5 μm , a three-
1072 fold zoom of a region framed in white containing a primary cilium is presented below the
1073 original images.

1074

1075 **Fig. 2. Identification of the microtubule binding domain in Sept9_i1. a** Top part: human
1076 Sept9_i1 specific amino-acid N-terminal sequence (1-24) was aligned with the homologous plant
1077 *A. thaliana* AIR9 sequences (173-205 and 46-78) contained in the AIR-9 microtubule binding
1078 domain (MBD) (Bushman et al. 2006); bottom part: Sept9_i1 and AIR9 sequences in top part
1079 were aligned with sequences of tandem repeats involved in the binding of human structural
1080 MAPs (Tau, MAP4 and MAP2) to microtubules (ex: Tau 4R isoform positions of repeats are
1081 R1₂₄₂₋₂₇₃, R2₂₇₄₋₃₀₄, R3₃₀₅₋₃₃₅ and R4₃₃₆₋₃₆₇). Alignments were adjusted by introducing gaps and
1082 similar aa residues were manually coded, orange: common to AIR9 and MAPs; yellow: common
1083 to Sept9_i1, AIR9 and MAPs; light green: common to Sept9_i1 and MAPs; dark green: common
1084 to MAPs; dark blue: common to Sept9_i1 and AIR9; light blue: common to AIR9 repeats.
1085 Important serine residues and hydrophobic residues of MAPs involved in MAP repeat-
1086 microtubule lattice interactions are framed in red and marked with a red asterisk, respectively.
1087 Horizontal blue line and red line indicate, respectively, the position of Tau R2 repeat and Tau
1088 R1/MAP4 R1 repeat modeled based on the cryo-EM structural determination of MAP repeated
1089 sequences bound on microtubules *in vitro* (¹Kellogg et al., 2018, ²Shigematsu et al., 2018). **b**
1090 Schematic representation of the Sept9_i1 mutants in the predicted MBD tagged with GFP at their
1091 C-terminus. **c**, **d** Co-localization of each Sept9 construct transfected in U2OS cells with
1092 microtubules or stress fibers during interphase was analyzed as described in Fig. 1 legend.
1093 Unpaired, two-tailed t-test with Welch's correction, ** $p < 0.01$, Microtubule and Actin
1094 localization vs Sept9_i1 wt; white scale bars 5 μm , insets: three-fold zooms. **e** Schematic
1095 representation of Sept9 isoforms (Sept9_i1, _i5 and _i3), of the chimeric construct fusing the
1096 Sept9_i1 MBD to the N-terminus of Sept9_i5 (Sept9_i1-i5) and of Sept9_i1 HNA mutants, all
1097 tagged with GFP at their C-terminus. **f** Co-localization of Sept9_i5 or _i1-i5 or _i3, and **g** of
1098 Sept9 HNA point mutants with microtubules or stress fibers in U2OS transfected cells during
1099 interphase. Unpaired, two-tailed t-test with Welch's correction, ** $p < 0.01$. **h** Images showing

1100 co-localization of Sept9 in U2OS cells transfected with constructs described in **e**; white scale
1101 bars 10 μm , insets: 1.4-fold zooms.

1102
1103 **Fig. 3. Dependence of septin association with microtubules on the incorporation of Sept9_i1**
1104 **in polymerized septin octamers.** **a** Top, predicted septin complexes when Sept9_i1 wt, or
1105 Sept9_i1 mutated on its G (G_{mut} , blue dots) or NC interface (NC_{mut} , yellow dot) is expressed in
1106 cells; bottom, Western blots of native gels resolving septin complexes present in U2OS cells
1107 expressing indicated siRNA and Sept9_i1 wt or Sept9_i1 interface mutants. **b** GFP-fluorescence
1108 based co-localization of Sept9_i1 wt or interface mutants with actin and microtubules; Sept9_i1
1109 wt co-localized both with microtubule and actin fibers as expected, but non-octameric septin
1110 complexes containing an interface mutant Sept9_i1 did not. White scale bar 10 μm , insets: two-
1111 fold zooms of frames region. **c** Top, predicted septin complexes when Sept2 wt or Sept2 mutated
1112 on its NC interface (NC_{mut} , yellow dots) is co-expressed with Sept9_i1 in U2OS cells; bottom,
1113 Western blots of native gels resolving septin complexes present in U2OS expressing indicated
1114 siRNAs and septin constructs. **d** mApple and GFP-based co-localization of Sept2 and Sept9_i1,
1115 respectively, with actin and microtubules; expression of Sept2 NC mutant prevented normal co-
1116 localization of octamers harboring Sept9_i1 with acetylated microtubules and actin fibers. Insets:
1117 individual fluorescence channel images of framed regions.

1118
1119 **Fig. 4. Sept9_i1 selective binding on, and stabilization of, microtubule bundles.** **a**
1120 Differential co-localization of endogenous Sept7 vs MAP4 with microtubule cytoskeleton in
1121 SKBr3 cells. **b** Differential co-localization between endogenous MAP4 and transfected
1122 Sept9_i1-GFP or Sept9_i1-i5-GFP with microtubule cytoskeleton in U2OS cells. White scale
1123 bars 5 μm in merge images. **c** Analysis of the percent of U2OS cells KD for Sept9 and
1124 expressing the indicated Sept9-GFP constructs, displaying either microtubule or actin or mixed
1125 cytoskeleton co-localization with GFP during interphase, and treated either with DMSO vehicle
1126 alone (D) or 2 μM paclitaxel (P) or 10 μM nocodazole (N) for 2 hrs. Results are from the
1127 analysis on a total of 90 cells from three independent experiments based (30 cells per
1128 experiment). Unpaired, two-tailed t-test with Welch's correction, ** $p < 0.01$, microtubule and
1129 actin localization vs DMSO. **d** Quantification of nocodazole-resistant acetylated microtubules
1130 (treatment as in **c**) in U2OS cells KD for Sept9 and expressing the indicated Sept9-GFP
1131 constructs. Results are from the analysis of a total of 150 cells from three independent
1132 experiments (50 cells per experiments). Unpaired, two-tailed t-test with Welch's correction, *
1133 $p < 0.05$, *** $p < 0.0005$.

1134
1135 **Fig. 5. Dependence of Sept9_i1 binding to microtubules on their bundling but not on their**
1136 **acetylation or polyglutamylation.** **a** Left: Western blots of total protein extracts from indicated
1137 cell lines, probed for Sept9_i1, α -tubulin acetylation on K40, glutamylation (one or two
1138 glutamate residues) and polyglutamylation of tubulin; as a positive control for these tubulin
1139 posttranslational modifications, an equivalent amount of purified porcine brain tubulin (Brain
1140 tub) was included in the last lane. Right: quantification of tubulin acetylation levels in cell lines
1141 based on acetylated α -tubulin and total α -tubulin Western blots. Results are from three
1142 independent determinations. Unpaired, two-tailed t-test with Welch's correction, * $p < 0.05$, ** $p <$
1143 0.01 , tubulin acetylation levels vs ARPE19. **b** Left: Co-localization by immunocytochemistry of
1144 Sept7 with microtubules and/or actin fibers in RPE1 and ARPE19 cells during interphase; white
1145 scale bars 10 μm , insets: two-fold zooms of framed regions. Right: quantification of this co-

1146 localization from three independent experiments on a total of 90 cells (30 cells per experiments).
1147 **c** Left: Co-localization by immunocytochemistry of Sept7 with microtubules bundles induced by
1148 paclitaxel treatment (PTX) and/or with actin fibers in RPE1 and ARPE19 cells; white scale bar
1149 10 μ m, insets: two-fold zooms of framed regions. Right: Western blots as described in **a** for total
1150 protein extracts of cells that were treated with vehicle alone (DMSO) or PTX. **d** Left: Co-
1151 localization by immunocytochemistry of Sept7 with microtubules bundles induced by paclitaxel
1152 treatment (PTX) in RPE1 cells either transfected with a control siRNA (siCtrl) or a siRNA
1153 against the α -tubulin acetyl transferase 1 (α TAT1); white scale bar 10 μ m. Right: Western blot of
1154 total protein extracts from PTX treated RPE1 cells as described in the left part, showing down-
1155 regulation of α -tubulin acetylation upon α TAT1 KD.

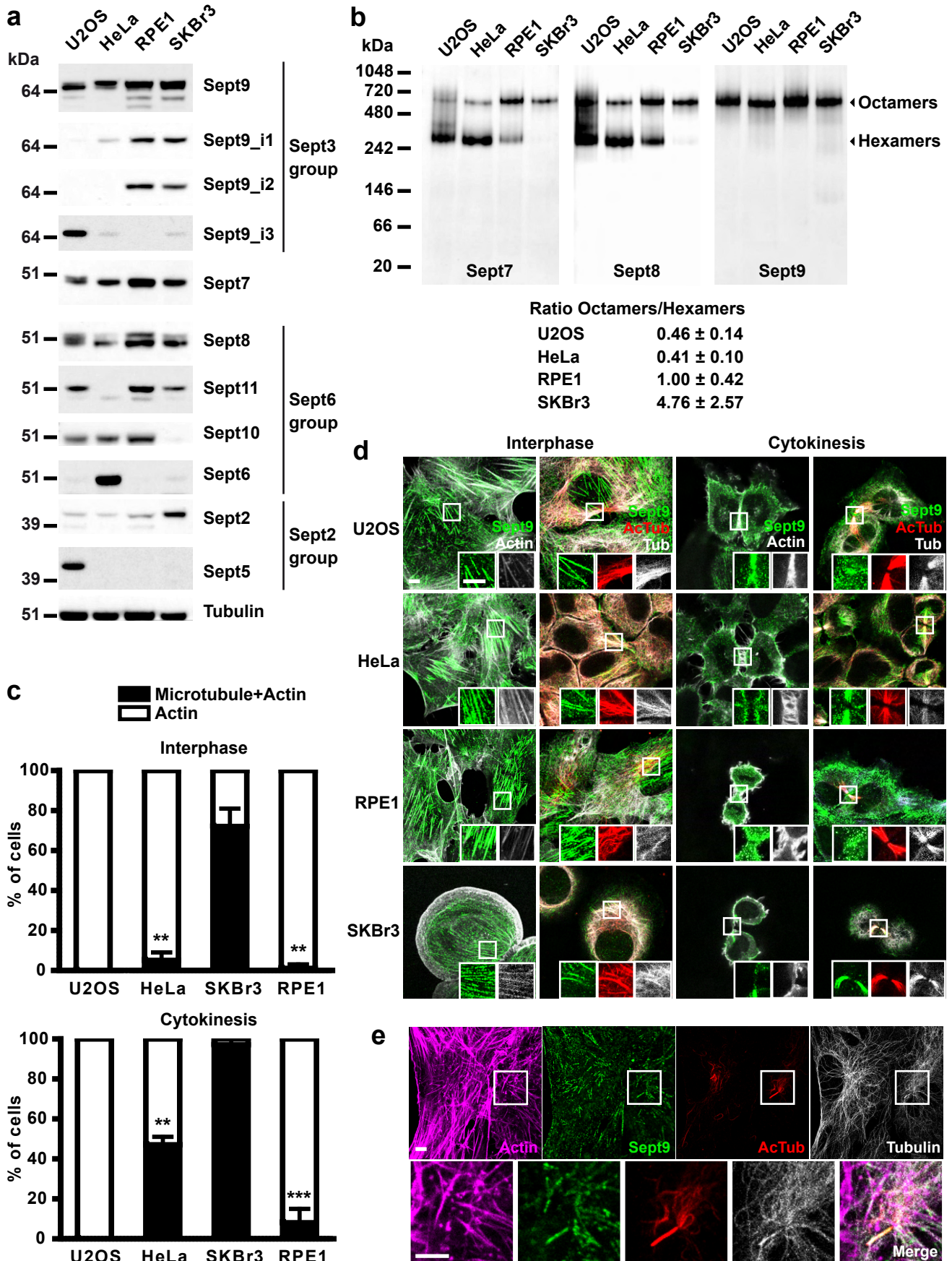
1156
1157 **Fig. 6. Recombinant septin octamers containing Sept9_i1 specifically bind microtubules *in***
1158 ***vitro*.** **a** Schematic representation of the *in vitro* assay. Biotinylated GMPCPP-stabilized
1159 microtubule seeds were immobilized on a streptavidin coated passivated glass coverslip, and free
1160 tubulin heterodimers (10 μ M) along with septin octamers (nM concentrations as indicated
1161 containing 10% msfGFP-labeled septins) were added. The seeds were labelled with a higher
1162 percentage (30%) of rhodamine-tubulin heterodimers than the free tubulin added (3-6%) to be
1163 able to distinguish the seed from the dynamic microtubules while using the same fluorophore. **b**
1164 Septin octamers harboring either Sept9_i1 or _i3 subunits (Oct_9i1 or Oct_9i3) were introduced
1165 in the chamber at the indicated concentrations and their binding observed on dynamic
1166 microtubules (examples of sporadic binding indicated by white arrow heads) and stable
1167 microtubule seeds (examples indicated by yellow arrow heads). White scale bar 5 μ m. **c**
1168 Examples (#1 and #2) of representative kymographs of microtubules observed as in **b** in the
1169 presence of the minimal (10 nM) or maximal (300 nM) septin octamer concentrations. False
1170 colors for rhodamine (Tub) and GFP (Sept2) fluorescence signals were used to optimize visually
1171 their merged image (third image from the left of each condition). Horizontal white scale bar 5
1172 μ m; vertical white scale bar 50 sec.

1173
1174 **Fig. 7. Recombinant septin octamers containing Sept9_i1 reduce the shortening rate of**
1175 **dynamic microtubules *in vitro*.** **a** Quantitation of microtubule dynamic parameters (growth rate,
1176 shortening rate, and catastrophe time frequency) at increasing septin octamer concentrations.
1177 Results are presented as box and whiskers plots superposed with individual data points including
1178 outliers. The number of microtubules used (n) and mean value are indicated under each plot.
1179 (grey: no septin octamers, pink: octamers with Sept9_i1, light blue: octamers with Sept9_i3).
1180 Two-tailed t-test with Benjamini & Hochberg p-value correction with R, * p<0.05, ** p<0.01,
1181 *** p<0.001, **** p<0.0001. Right inset graphs show median values of each parameter in the
1182 absence of octamers (black dots) and direct comparison in presence of either octamers with
1183 Sept9_i1 (red dots) or octamers with Sept9_i3 (blue dots) and corresponding interquartile ranges.
1184 **b** Representative kymographs (rhodamine channel for tubulin) representing the effect of
1185 recombinant septin octamers on microtubule dynamics. Such kymographs were used to calculate
1186 the parameters values presented in **a** from individual microtubules. First kymograph on the left is
1187 in the absence of septin octamers. Horizontal white scale bar 5 μ m; vertical white scale bar 50
1188 sec. **c** Snapshots at indicated times (min:sec) of a microtubule and associated septin octamers
1189 harboring Sept9_i1 (200 nM, Supplementary Movie 3). Yellow arrow heads point at a curved
1190 structure on a depolymerizing microtubule (+) end. White scale bar 5 μ m.

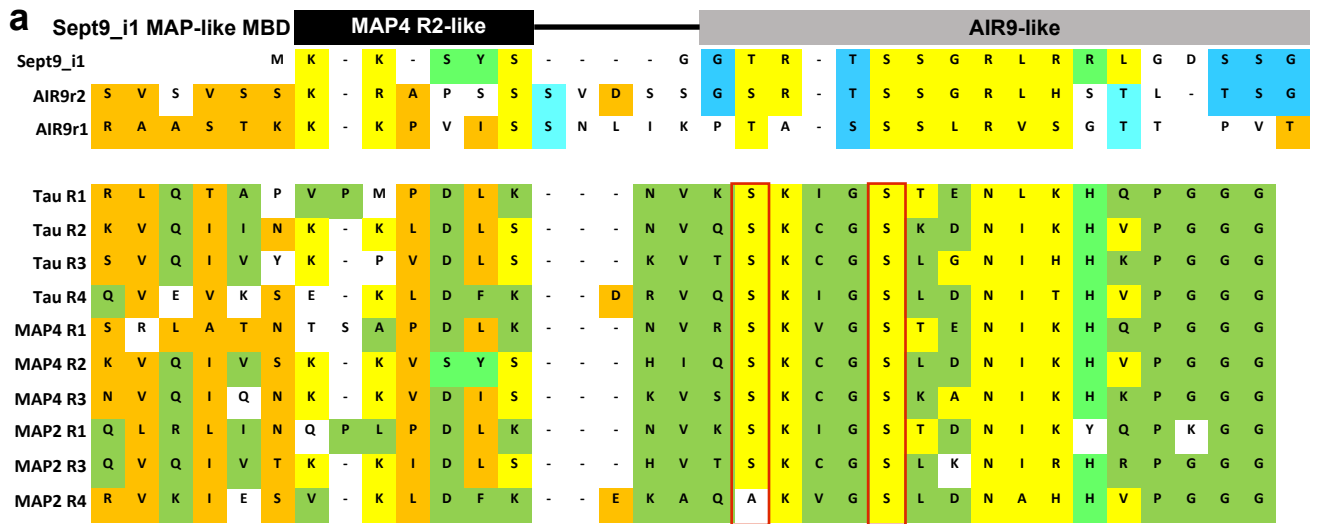
1191

1192 **Fig. 8. Impact on actin cytoskeleton and cell shape of septin octamer sequestration by**
1193 **microtubules via Sept9_i1 MBD. a** Analysis of the presence of sub-nuclear actin fibers in
1194 U2OS transfected by either siCtrl or siSept9. Results are from three independent experiments on
1195 a total of 90 cells per condition (30 cells per experiments). Unpaired, two-tailed t-test with
1196 Welch's correction, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.0005$, % of cells vs siCtrl. Right: Co-
1197 localization of Sept7 with sub-nuclear actin stress fibers. White scale bar 10 μm , insets: two-fold
1198 zooms of frames region. **b** Analysis of the presence of sub-nuclear actin fibers in U2OS derived
1199 cell lines stably expressing each one of the indicated C-terminally GFP tagged Sept9_i1
1200 constructs and the histone mCherry-H2B fusion. Results are from a single determination on 100
1201 cell images per cell lines. **c** Analysis of shape changes on derived U2OS cell lines described in **b**
1202 and adhering to medium size H micropatterns. d1: average maximum width of 35.170 μm
1203 observed from measurement on four empty H micropatterns; d2: observed width of single
1204 adherent cells at mid-maximal height of the H micropatterns. Representative images of merged
1205 fluorescence channels of the three U2OS derived cell lines selected for analyses. Note that as for
1206 the rest of the figure, these cell lines (U2OS.Sept9_i1-GFP.mCherry-H2B, U2OS.Sept9_i1-i5-
1207 GFP.mCherry-H2B and U2OS.Sept9_i1 Δ 1-25-GFP.mCherry-H2B) are indicated simply by the
1208 originally transfected Sept9-GFP construct names. White scale bar is 10 μm . Right:
1209 measurements of the difference between d1 and d2 in cells from selected lines showing a higher
1210 value when septins bind to microtubules, due to a reduction of the cell width at mid-maximal
1211 height. Results are from two independent experiments on a total of 120 cells (60 cells per
1212 experiment). Unpaired, two-tailed t-test with Welch's correction, *** $p < 0.0005$, d1-d2 vs
1213 Sept9_i1 Δ 1-25. Red lines represent mean values \pm SEM. **d** Measurements of d1-d2, as described
1214 in c, in U2OS cells transfected either by either siCtrl or siSept9 as described in c showing that
1215 Sept9 KD reduced cell width at mid-maximal height. Results are from three independent
1216 experiments on a total of 120 cells (40 cells per experiment). Unpaired, two-tailed t-test with
1217 Welch's correction, *** $p < 0.0005$, d1-d2 vs siCtrl. Red lines represent mean values \pm SEM.

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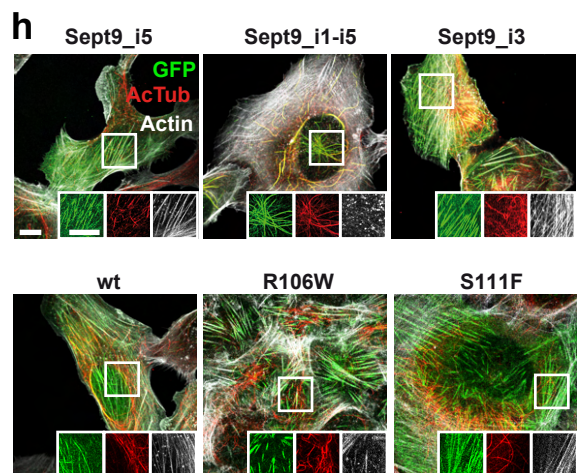
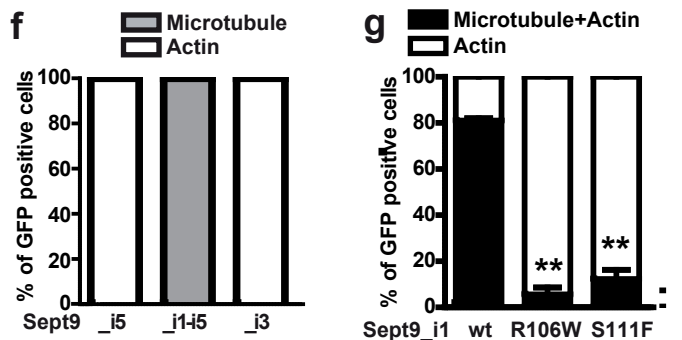
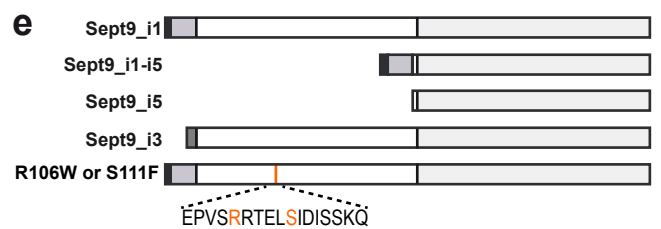
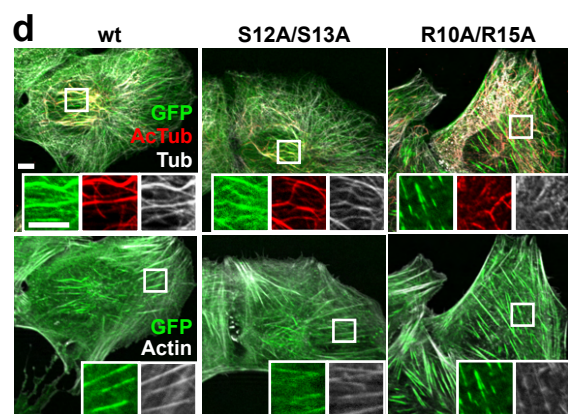
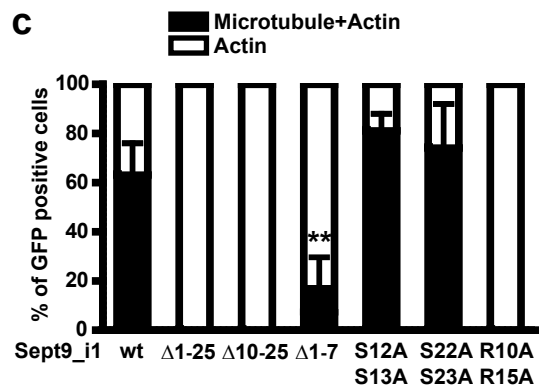
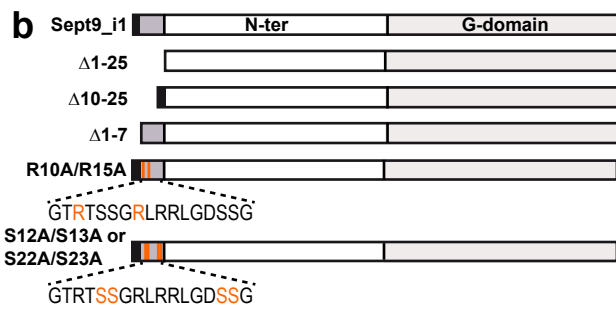


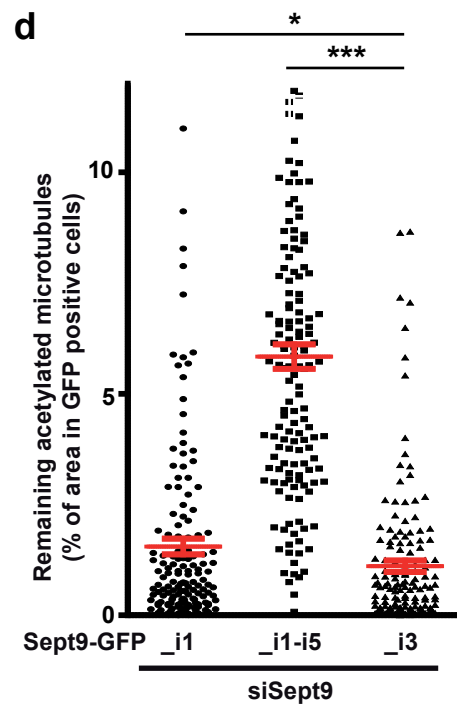
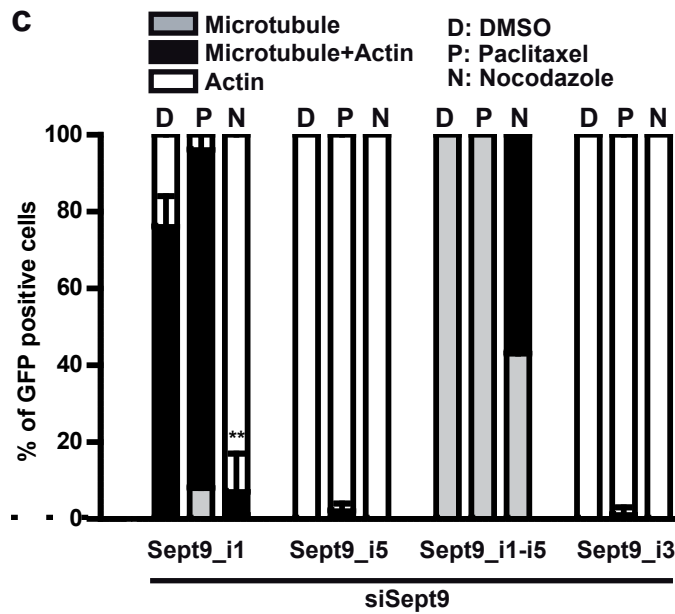
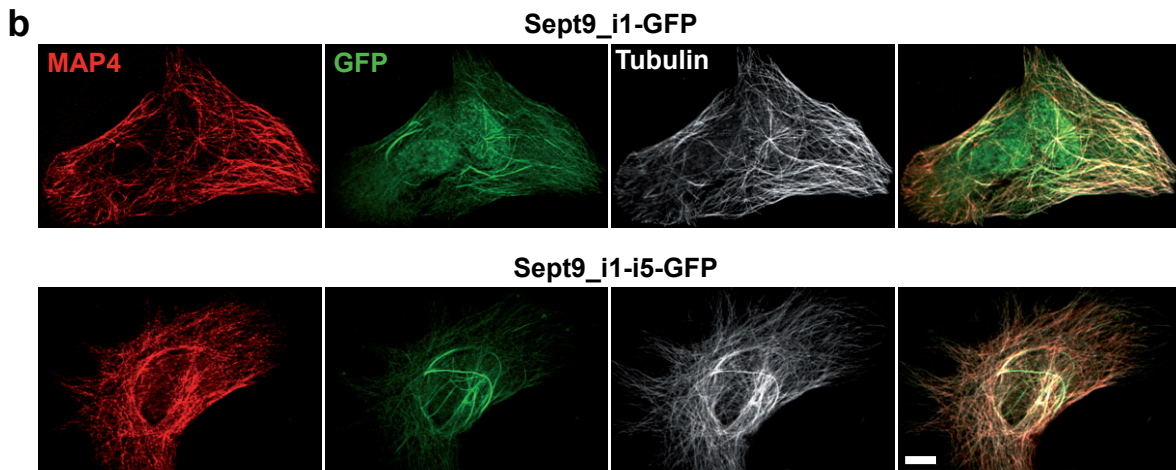
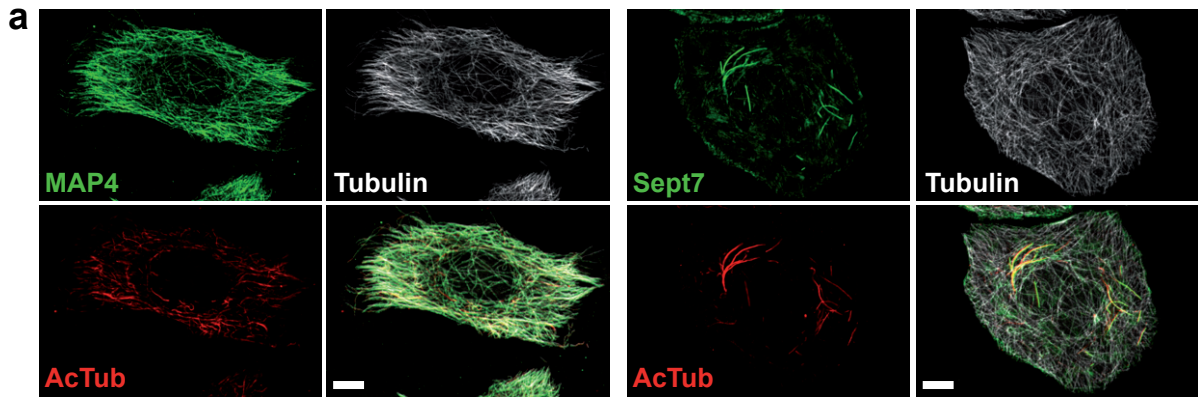
Kuzmic et al. Figure 1

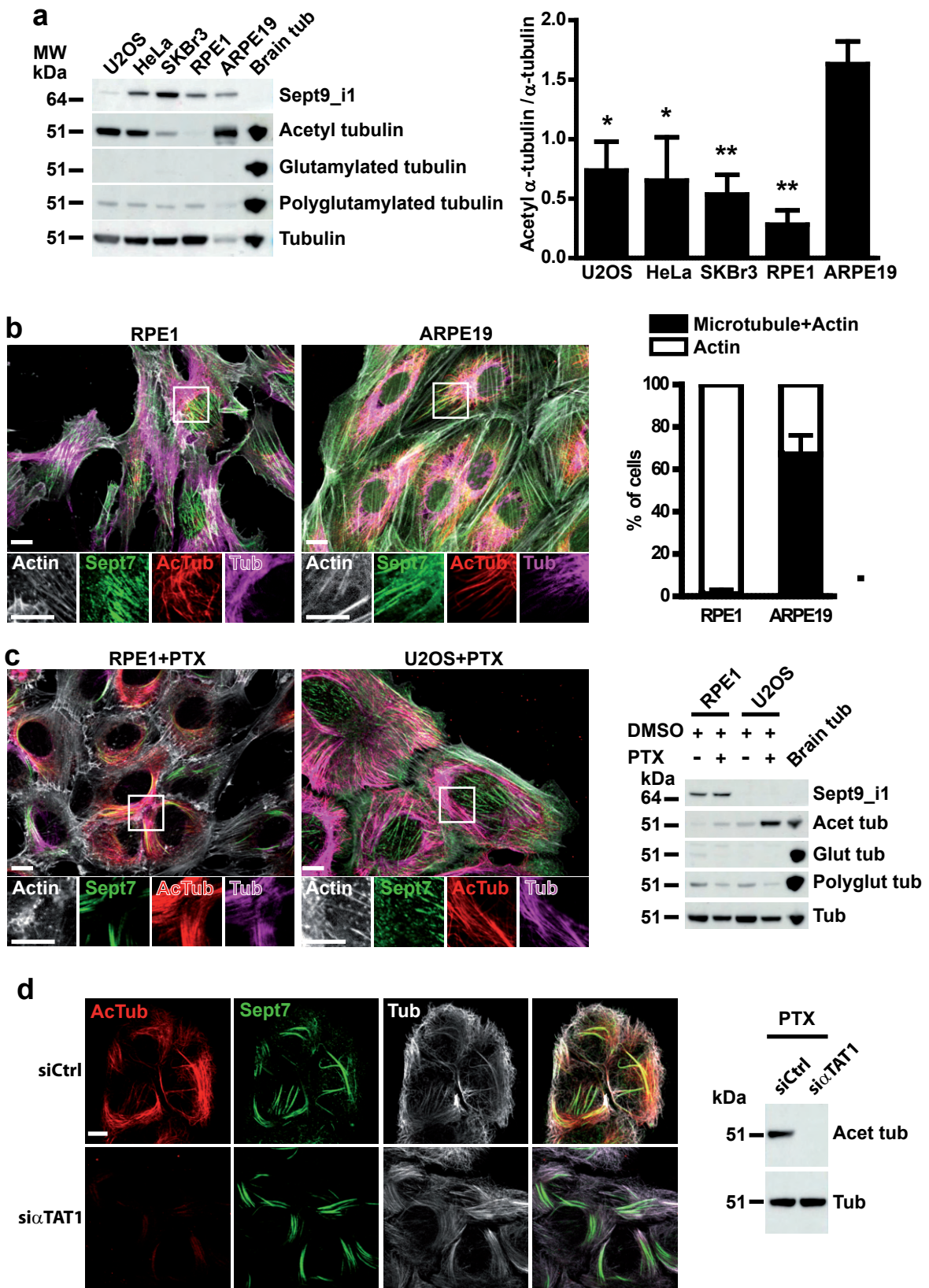


modeled Tau R2¹

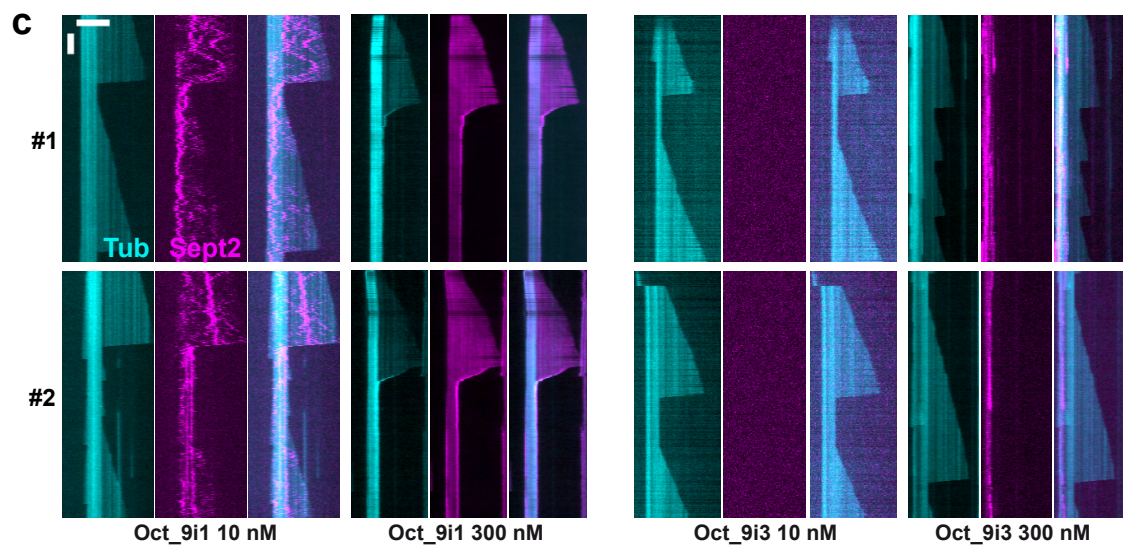
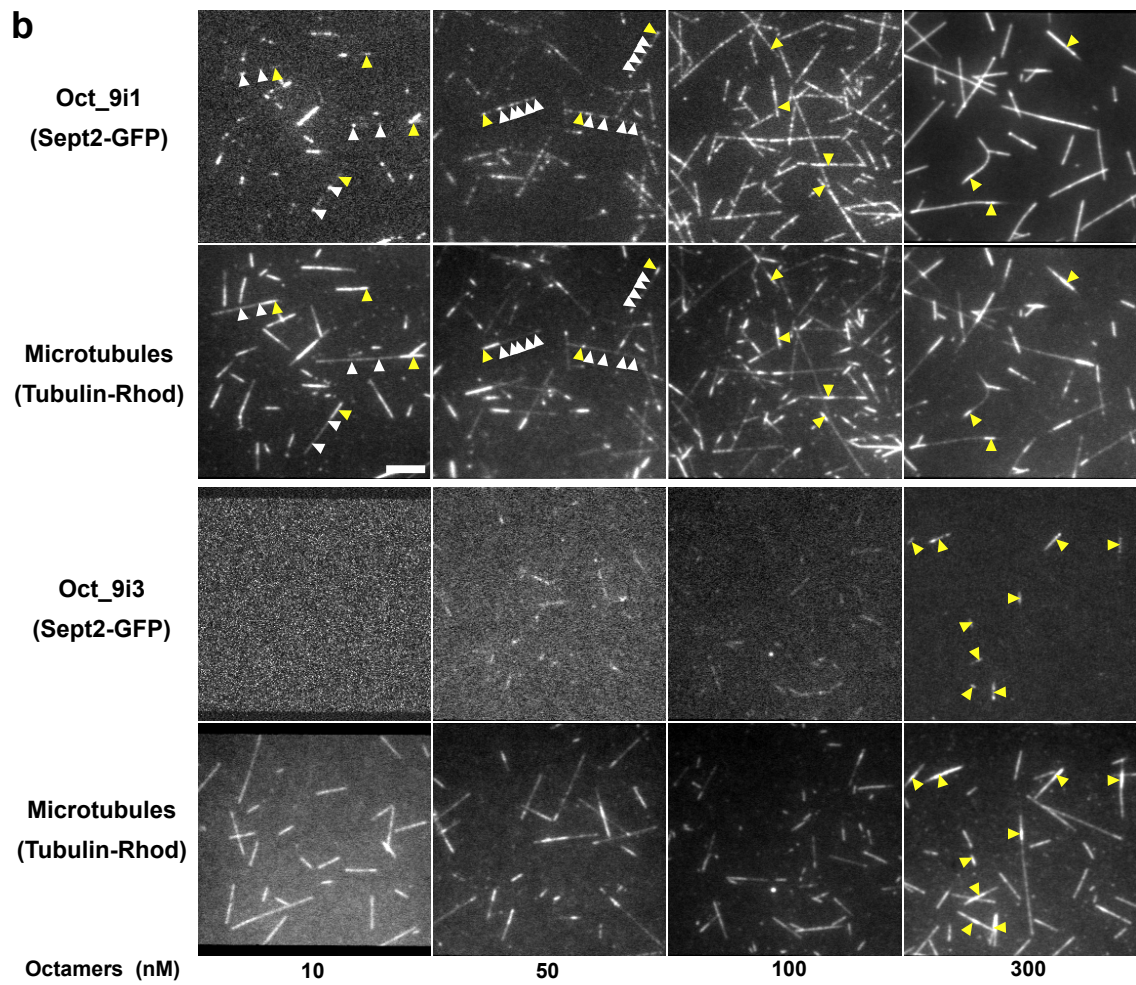
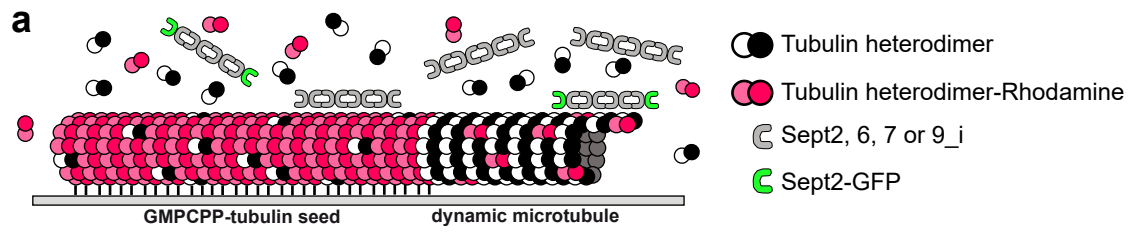
modeled Tau R1¹ or MAP4 R1²

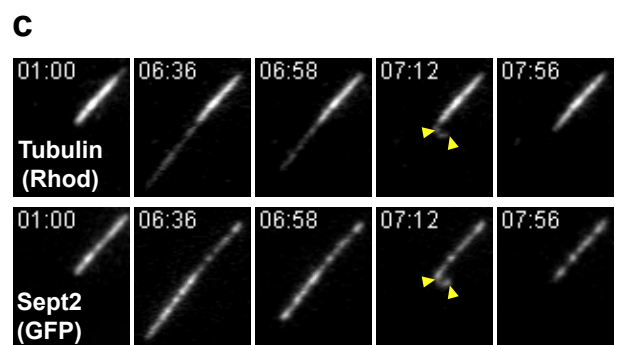
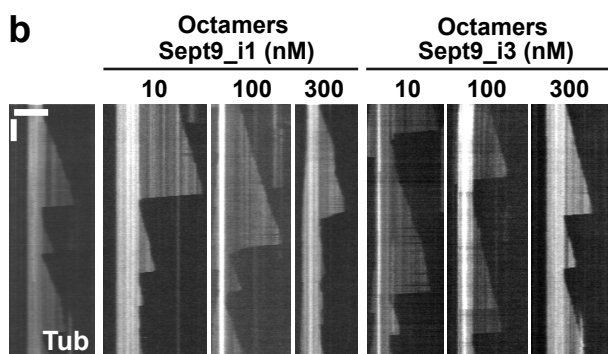
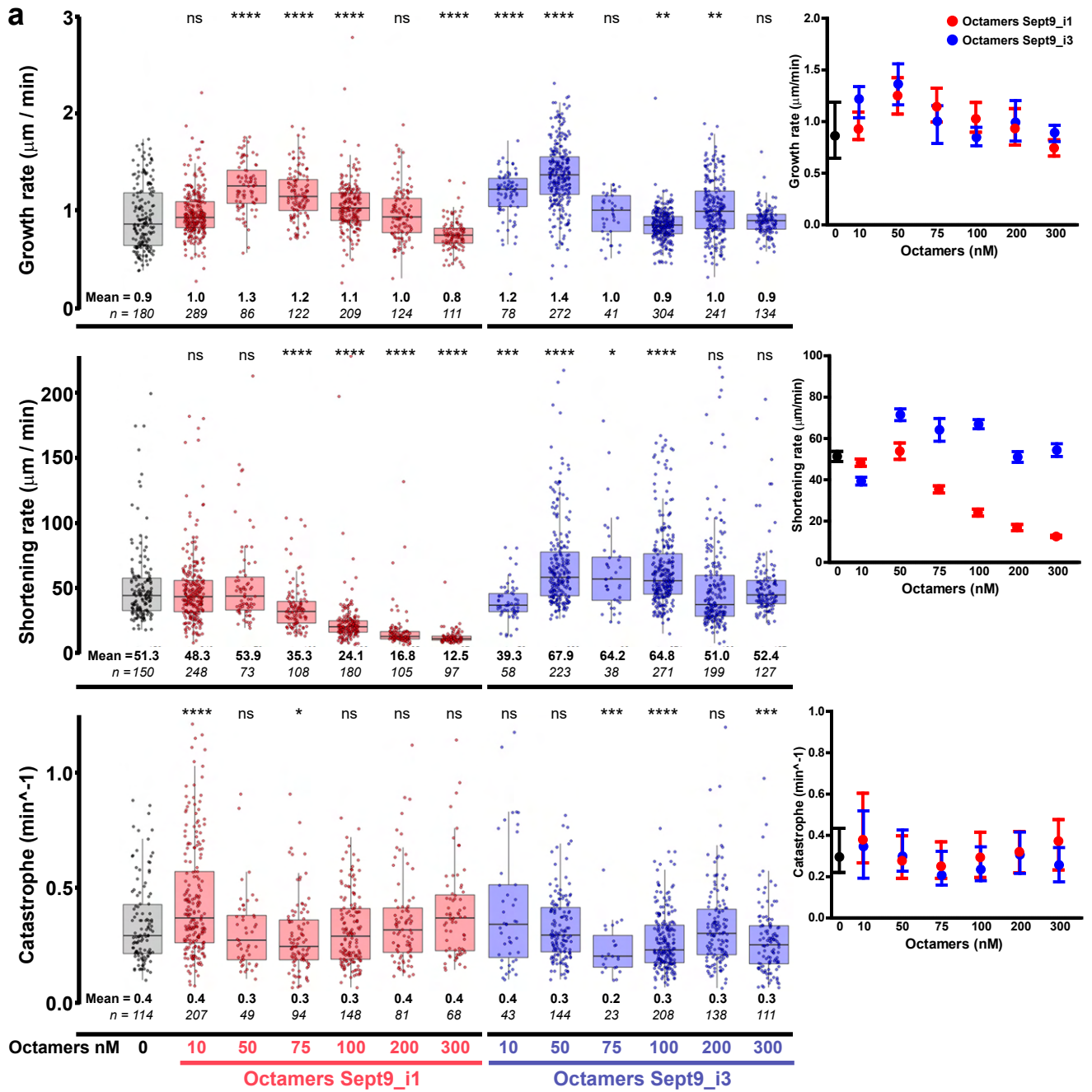




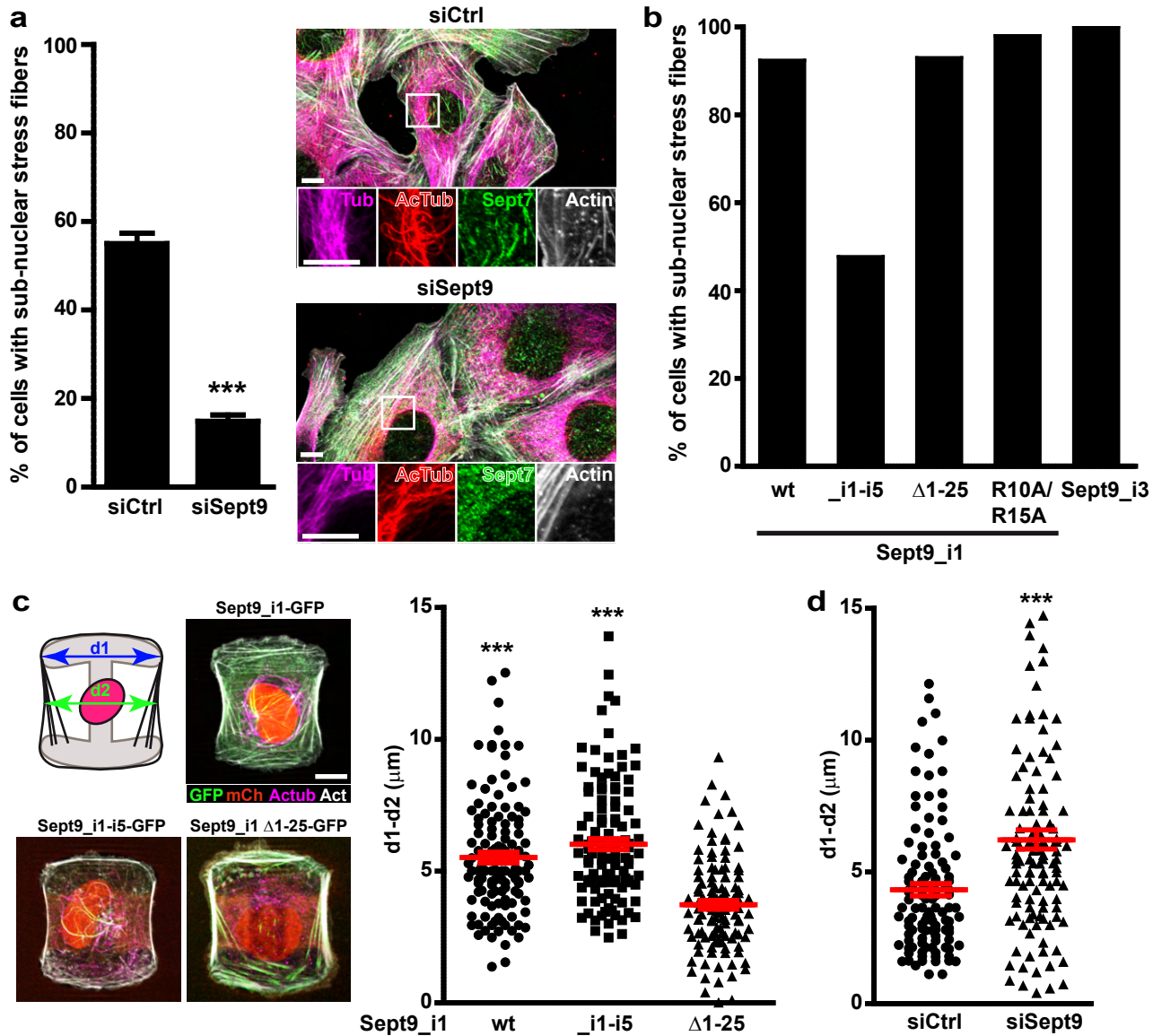


Kuzmic et al. Figure 5





Kuzmic et al. Figure 7



Kuzmic et al. Figure 8

1238 **Supplementary Figure legends**

1239 **Supplementary Fig. 1. Separation of septin complexes on native blue gels and detection by**
1240 **Western blot. a** Relation between migration distances of native septin complexes from the top
1241 of the 4-16% acrylamide gradient gels and those of native MW markers; each data point
1242 represents the average of two or three independent determinations. Right: table presenting
1243 observed apparent MW and theoretical MW of the different complexes. **b** Recombinant septin
1244 hexamers and octamers were run on gradient native gels as in **a** and were detected by Coomassie
1245 blue staining; the asterisk indicates the migration position of putative septin monomers. **c** Native
1246 protein extracts from Hela cells were run on native gels and blotted on a PVDF membrane;
1247 septin hexamers and octamers were detected by WB. **d** U2OS cells were KD for Sept7 or Sept9
1248 and native extracts were analyzed as in **c**.

1249 **Supplementary Fig. 2. Protein sequence conservation of the Sept9_i1 MBD. a** Alignment of
1250 Sept9_i1 sequences grouped by phylogenetically closely related organisms (birds and reptiles
1251 highlighted in green) and mammals (quadrupeds in blue, rodents in purple, and primates in
1252 turquoise). Amino acid residues are highlighted based on Clustal X color coding. A conservation
1253 score histogram for each position is presented below the alignments of sequences; under each
1254 column is indicated the numerical index reflecting the conservation of physico-chemical
1255 properties of amino acid residues (asterisk : 100% identical residues, plus sign: alternative
1256 residues with conserved properties). Columns exhibit a color shading coding from the maximal
1257 conservation index (11=asterisk) in pure yellow to the minimal conservation index (2) in dark
1258 brown. Sept9_i1 1-25 sequences were recovered using the PSI-BLAST tool and the human
1259 sequence as the seed, imported in Jalview, aligned with Mafft (redundant and truncated
1260 sequences were eliminated), and grouped by phylogenic proximity by BLOSUM52; the two
1261 highly conserved arginine residues R10 and R15 mutated in this study are indicated at the top of
1262 alignments. **b** Consensus logo of the Sept9_i1 MBD was generated using Jalview.

1263 **Supplementary Fig. 3. Cellular localization of Sept9 by immunocytochemistry. a**
1264 Fluorescent detection of Sept9 and tubulin on Western blots of total protein extracts from U2OS
1265 cells transiently transfected by a control siRNA (siCtrl) or co-transfected with an siRNA against
1266 the 3'UTR region of Sept9 mRNA (siSept9) and with respective constructs described in Fig 2b.
1267 Numbers at the bottom of each lane indicate the levels of expression of Sept9 constructs relative
1268 to those of endogenously expressed Sept9 in first lane as quantified on this WB. **b** Percentages of
1269 U2OS cells transfected with the indicated Sept9-GFP constructs displaying co-localization of
1270 Sept9 with microtubules and actin fibers or only actin fibers during cytokinesis. Percentages
1271 were determined from three independent experiments based on a total of 90 cells (30 cells per
1272 experiment). Unpaired, two-tailed t-test with Welch's correction, * $p < 0.05$, microtubule and
1273 actin localization vs Sept9_i1 wt. **c** U2OS cells transfected with the indicated Sept9-GFP
1274 constructs in cytokinesis. Insets are two-fold zoomed regions framed by a white square in the
1275 corresponding original images. **d** Percentages of U2OS cells transfected with the indicated
1276 Sept9-GFP constructs displaying co-localization of Sept9 with microtubules and actin fibers or
1277 only actin fibers during cytokinesis. Percentages were determined from three independent
1278 experiments based on a total of 90 cells (30 cells per experiment). Unpaired, two-tailed t-test

1279 with Welch's correction, ** $p < 0.01$, microtubule and actin localization vs Sept9_i5. **e** U2OS
1280 cells expressing Sept9_i5-GFP. Insets are 2.5-fold zoomed regions framed by a white square in
1281 the corresponding original images. **f** Comparison of percentages of cells displaying co-
1282 localization of Sept9 with microtubules and actin fibers or only actin fibers during cytokinesis in
1283 RPE1 and ARPE19 cell lines. Percentages were determined from three independent experiments
1284 based on a total of 90 cells (30 cells per experiment). Unpaired, two-tailed t-test with Welch's
1285 correction. **g** U2OS cells transfected with the indicated Sept9-GFP constructs and treated either
1286 by vehicle (DMSO) or 10 μM nocodazole for two hours.

1287 **Supplementary Fig. 4. Characterization of U2OS cell lines stably co-expressing Sept9-GFP**
1288 **and mCherry-H2B constructs.** **a** Expression of Sept9-GFP constructs (Sept9_X-GFP) and total
1289 endogenous Sept9 (top Western blot) and expression of Sept9_i3-GFP constructs and
1290 endogenous Sept9_i3 (bottom Western blot) in U2OS cell lines stably co-expressing mCherry-
1291 H2B and Sept9-GFP constructs (U2OS.Sept9_X-GFP.mCherry-H2B abbreviated here as
1292 U2OS.Sept9_X-GFP) and in the non-transfected parental U2OS cell line. **b** Percentages of cells
1293 displaying co-localization of Sept9_X-GFP with microtubules and actin fibers in
1294 U2OS.mCherry-H2B.Sept9_X-GFP cell lines. Results are from one determination in 30 cells
1295 from each cell line. **c** Images of Sept9_X-GFP constructs localization in U2OS.Sept9_X-
1296 GFP.mCherry-H2B cell lines, mCherry-H2B and acetylated tubulin fluorescence signals are
1297 shown in false blue and red colors, respectively.

1298 **Supplementary Fig. 5. Phenotyping cells based on the presence of sub-nuclear actin fibers.**
1299 **a** Top and bottom view of actin fibers (phalloidin-Atto 390), Sept9_i1 (GFP), and nucleus
1300 (mCherry-H2B) in a U2OS.Sept9_i1-GFP.mCherry-H2B cell. White scale bar 10 μm . **b** Effects
1301 of KD of Sept7 or Sept9 in RPE1 cells on the relative expression of Sept7 and Sept9 and **c** on the
1302 presence of hexamers and octamers. **d** presence of sub-nuclear actin fibers in RPE1 cells KD for
1303 Sept9, i.e. in the absence of octamers. In **b**, Western blot signals of Sept7 or Sept9 expression
1304 were first normalized to the respective Western blot signals of α -tubulin and then further
1305 normalized to the ratio obtained from cells transfected with Ctrl siRNA; results are from three
1306 independent experiments. Paired, two-tailed t-test with Welch's correction, ** $p < 0.01$, ***
1307 $p < 0.0005$. In **c**, Western blots of native extracts resolved on native 4-16% acrylamide native gels.
1308 In **d**, results are from three independent experiments on a total of 90 cells (30 cells per
1309 experiment). Unpaired, two-tailed t-test with Welch's correction, ** $p < 0.01$, % of cells vs siCtrl.

1310 **Supplementary Table 1. Oligonucleotide primer sequences used for the generation of the**
1311 **plasmids used in this study.** Oligonucleotides marked with an asterisk are shared among several
1312 constructs, but are listed only once.

1313 **Supplementary Movie 1. Dynamics of septin octamers harboring Sept9_i1 (Oct_9i1)**
1314 **binding with microtubules *in vitro*.** Series of videos corresponding to each of the 10 to 300 nM
1315 of Oct_9i1 that were used. White scale bar 5 μm .

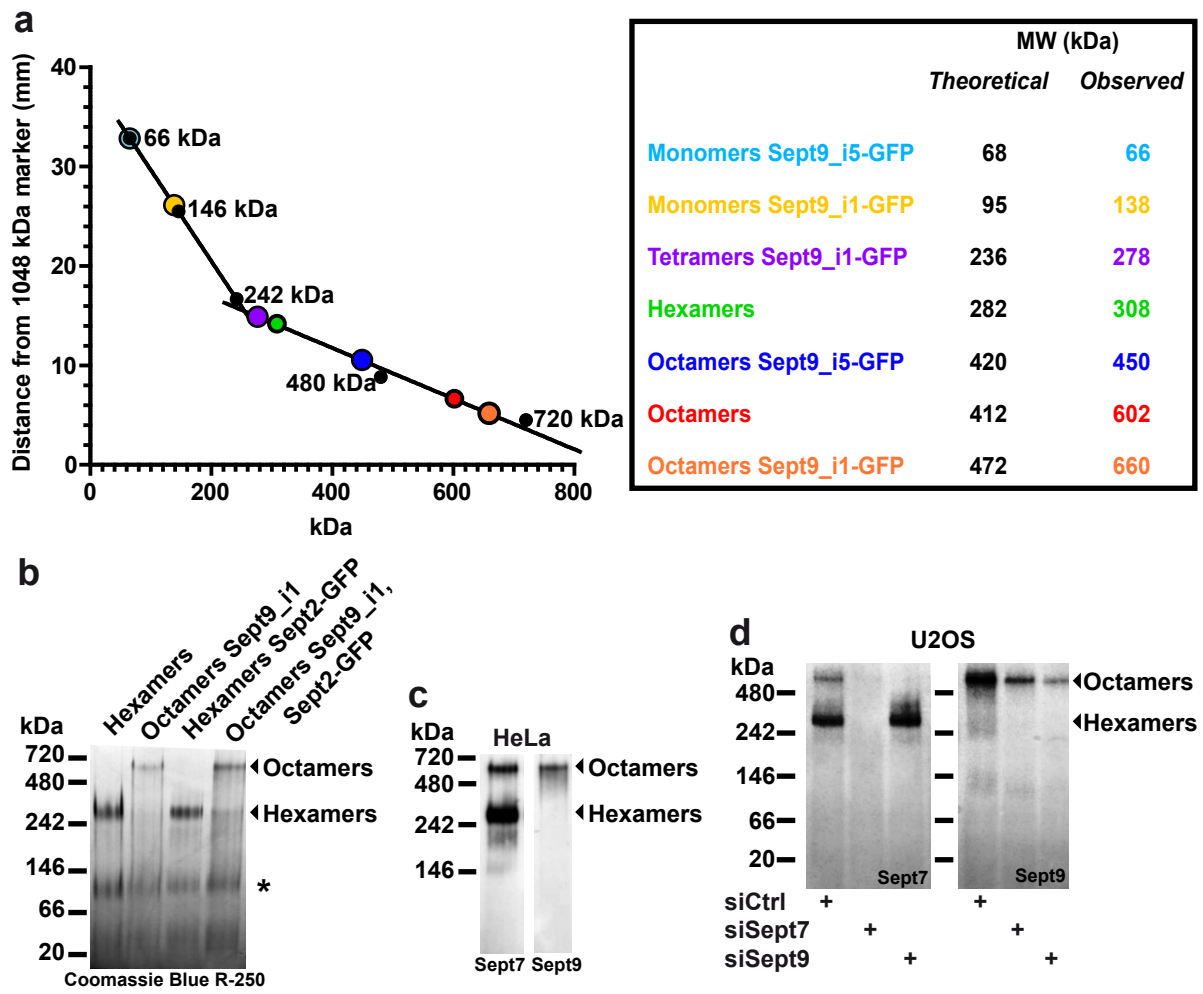
1316 **Supplementary Movie 2. Dynamics of septin octamers harboring Sept9_i3 (Oct_9i3)**
1317 **binding with microtubules *in vitro*.** Series of videos corresponding to each of the 10 to 300 nM
1318 of Oct_9i3 that were used. White scale bar 5 μm .

1319 **Supplementary Movie 3. Depolymerizing microtubules (examples 1 and 2) in presence of**
1320 **200 nM of septin octamers harboring Sept9_i1 (Oct_9i1) *in vitro*.** Series of videos
1321 corresponding to each example. White scale bar 5 μm .

1322

promoter	in-house plasmid name	plasmid	primer name	primer sequence (5' to 3')	primer length	
CMV			Sept9_i1-msfGFP	NheISept9_i1FOR	CGTCAGATCCGCTAGCATGAAGAAGTCTTACTCAGGAGGCAC	42
			Sept9_i3-msfGFP	NheISept9_i3FOR	CGTCAGATCCGCTAGCATGGAGAGGGACCGGATCTC	36
				Sept9sfGFPREV	TTGGACACCATCTCTGGGGCTTCTGGCTCC	30
				Sept9sfGFPFOR	AGAGATGGTGTCCAAGGGCGAGGAGC	26
				BamHIsgFPREV	TAGATCCGGTGGATCCTTACTGTACAGCTCATCCATGCC	40
CMV		Sept2-mApple	NheISept2FOR	CGTCAGATCCGCTAGCATGTCTAAGCAACAACCAACTCAGT	41	
			hSept2mAppleREV	CTCGCCCTTGCTCACCACATGGTGGCCGAGAGC	33	
			mAppleFOR	GTGAGCAAGGGCGAGGAG	18	
			BamHI mAppleREV	TAGATCCGGTGGATCCTCACTGTACAGCTCGTCCATGCC	40	
			NdeICMVpromoterFOR	ATCAAGTGTATCATATGCCAAGTACGCCCCCTATTGACGTCAATGACG	48	
CMV	pl-14	Sept2 F20D V27D-mApple	Sept2FDREV	GATTGGGAAGATTTGCATCTCCAACATAGCCAGGAGTTTCTGG	43	
			Sept2FDFOR	CAAATCTTCCAATCAAGATCACCGAAAATCAGTGAAGAAGGGGTTTCG	48	
			Sept9IDREV	TGCTCAGGATGGAGTCATCCCCACGTAGCCGAAGTCC	39	
CMV	pl-6	Sept9_i1-1281D M288D-msfGFP (Sept9_i1 NCmut2)	Sept9MDFOR	CTCCATCTGGAGCAGGATCGCCGGAAGCCATGAAGC	38	
			a0h_Sept9_REV	CGCCGCGCATCTGCTCCAGGATGGAGTCAATCC	35	
CMV	α0h-4	Sept9_i1 R289A R290A K291A K294A-msfGFP (Sept9_i1 NCmut1)	a0h_Sept9_FOR	CAGATGGCCGCGCGCCATGGCGCAGGGCTTCGAGTTCAACATCATG	48	
			Sept9i1W520AREV	AACTTCGATGGTACCCGCTTGGTCTTCTCCCAAGGATCC	41	
CMV	pl-12	Sept9_i1 W520A H530D-msfGFP (Sept9_i1 Gmut)	Sept9i1H530DFOR	GGTACCATCGAAGTTGAAAACACCACAGATTGTGAGTTGCCTACCTGCGG	51	
			NheISept9dNFOR	CGTCAGATCCGCTAGCATGGCCTTGAAAAGATCTTTTGAGGTCG	44	
CMV	is-7	Sept9_i5-msfGFP	NheISept9i5FOR	CGTCAGATCCGCTAGCATGGCCGACACCCCCAG	33	
CMV	is-9	Sept9_i1-i5-msfGFP	NheI9i1_Sept9i5FOR	CGTCAGATCCGCTAGCATGAAGAAGTCTTACTCAGGAGGCACGCGACCTCCAGTGCCCGGCTCCGGAGGCTTGGTGACTCCAGTGGCCAGCCGACACCCCCAGAGATG	110	
CMV	mt-1	Sept9_i1 Δ10-25-msfGFP	NheISept9i1D10FOR	CGTCAGATCCGCTAGCATGAAGAAGTCTTACTCAGGAGGCACGCGACCTCCAGTGCCCGCTCCGGAGGCTTGGTGACTCC	73	
CMV	mt-2	Sept9_i1 R10A R15A-msfGFP	NheISept9i1R10R15FOR	CGTCAGATCCGCTAGCATGAAGAAGTCTTACTCAGGAGGCACGCGACCTCCAGTGCCCGCTCCGGAGGCTTGGTGACTCC	82	
CMV	mt-5	Sept9_i1 S12A S13A-msfGFP	NheISept9i1S12S13FOR	CGTCAGATCCGCTAGCATGAAGAAGTCTTACTCAGGAGGCACGCGACCGCCGCGCCGGCTCCGGAGG	70	
CMV	mt-6	Sept9_i1 S22A S23A-msfGFP	NheISept9i1S22S23FOR	CGTCAGATCCGCTAGCATGAAGAAGTCTTACTCAGGAGGCACGCGACCTCCAGTGCCCGGCTCCGGAGGCTTGGTGACTCCAGTGGCCAGCCGACCTTGAAAAGATCTTTTGAGG	113	
CMV	mt-20	Sept9_i1 ΔN7-msfGFP (Sept9_i1 Δ1-7)	NheISept9i1DN7FOR	CGTCAGATCCGCTAGCATGGGCACGCGGACCTCCAG	36	
CMV	Hna-1	Sept9_i1 R106W-msfGFP	Sept9RWREV	CAGTGCCGACGACCCGGCTCGCC	26	
			Sept9RWFOR	TGCTTGGCGCACTGAGCTGTCCATTGAC	29	
CMV	Hna-2	Sept9_i1 S111F-msfGFP	Sept9SFREV	GATGTCAATGAACAGCTCAGTGCAGCGG	28	
			Sept9SFFOR	CTGTTTCATTGACATCTCGTCCAAGCAGGTG	30	

Supplementary Table 1. Oligonucleotide primer sequences used for the generation of the plasmids used in this study.

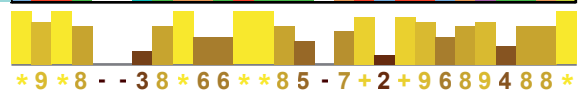


Kuzmic et al. Supplementary Figure 1

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		R10	R15	
XP_034645779.1 [<i>Trachemys scripta elegans</i>]	MKK	S-VHSGIARTSS	-GR	LRR LGDPTSP
XP_030390263.1 [<i>Gopherus evgoodei</i>]	MKK	S-VHSGISRTSS	-GR	LRR LGDPTSP
XP_024062065.1 [<i>Terrapene carolina triunguis</i>]	MKK	S-VHSGITRTSS	-GR	LRR LGDPTSP
XP_014457381.1 [<i>Alligator mississippiensis</i>]	MRK	S-SYSGARTSS	-GK	L R K A G D P T S P
XP_019398237.1 [<i>Crocodylus porosus</i>]	MRK	S-SYSGARTSS	-GR	L R K A G D P T S P
XP_015269831.1 [<i>Gekko japonicus</i>]	MRK	T-SYSGISRTSS	-GR	LRR LGNPTSP
XP_020641631.1 [<i>Pogona vitticeps</i>]	MRK	T-SYSGISRTSS	-GR	LRR LGDPTSP
XP_028572882.1 [<i>Podarcis muralis</i>]	MRK	T-SNTGISRTSS	-GR	LRR FGDPTSP
XP_026557860.1 [<i>Pseudonaja textilis</i>]	MKK	TSSYTGT SRTSS	-VR	LRR FGDPTSP
XP_026523333.1 [<i>Notechis scutatus</i>]	MKK	TSSYTGT SRTSS	-GR	LRR FGDPTSP
XP_008102562.1 [<i>Anolis carolinensis</i>]	MKK	T-SYTGISRTSS	-GR	LRR LGDPTSP
XP_021270206.1 [<i>Numida meleagris</i>]	MRK	S-MYSGITRTSS	-GR	LRR L S D Q T S P
XP_015735503.1 [<i>Coturnix japonica</i>]	MRK	S-TYSGITRTSS	-GR	LRR L S D Q T S P
XP_013034321.1 [<i>Anser cygnoides domesticus</i>]	MRK	S-SYSGITRTSS	-GR	LRR L S D Q T S P
XP_026716736.1 [<i>Athene cucularia</i>]	MRK	S-SYSGISRTSS	-GR	LRR L S D Q T S P
XP_030900675.2 [<i>Melopsittacus undulatus</i>]	MKK	S-SYSGISRTSS	-GR	P R R L S D Q T S P
XP_027741897.1 [<i>Melopsittacus undulatus</i>]	MKK	S-SYSGISRTSS	-GR	LGR L S D Q T S P
XP_029870538.1 [<i>Aquila chrysaetos chrysaetos</i>]	MKK	S-SYSGISRTSS	-GR	LRR L S D Q T S P
XP_014740523.1 [<i>Sturnus vulgaris</i>]	MKK	S-SYSGITRTSS	-GR	LRR L S E Q T S P
XP_025959670.1 [<i>Dromaius novaehollandiae</i>]	MKK	S-SYSGITRTSS	-GR	LQR L S D Q T S P
XP_026654321.1 [<i>Zonotrichia albicollis</i>]	MKK	S-SYSGITRTSS	-GR	LRR L S D Q T S P
XP_023387505.1 [<i>Pteropus vampyrus</i>]	MKK	S--YSGVTRTSS	-DR	L R K L G D P A G P
XP_025323914.1 [<i>Canis lupus dingo</i>]	MKK	S--SSGVTRTSS	-NR	L R K L G D P T G P
XP_016047785.1 [<i>Erinaceus europaeus</i>]	MKK	S--SSGV SRTSS	-SR	L R R L G D P S G P
XP_027705848.1 [<i>Vombatus ursinus</i>]	MKK	S-SGSGVMRTS	I-SR	-RR FGD T SGP
XP_020821661.1 [<i>Phascolarctos cinereus</i>]	MKK	S-SGSGVMRTS	I-SR	-RR L G D T SGP
XP_017506039.1 [<i>Manis javanica</i>]	MKK	S--SSGVTRTSS	-SR	L R R L G D L S G P
XP_025715323.1 [<i>Callorhinus ursinus</i>]	MKK	S--YSGVTRTSS	-NR	L R K L G D T T G P
XP_012415706.1 [<i>Odobenus rosmarus divergens</i>]	MKK	S--YSGVTRTSS	-NR	L R K L G D A T G P
XP_026339835.1 [<i>Ursus arctos horribilis</i>]	MKK	S--YSGVTRTSS	-NR	L R K L G E P T G P
XP_023099861.1 [<i>Felis catus</i>]	MKK	S--YSGVTRTSS	-NR	L R K L G D P T G P
XP_032726397.1 [<i>Lontra canadensis</i>]	MKK	S--YSGVTRTSS	-NR	L R K L G D P S G P
XP_007185825.1 [<i>Balaenoptera acutorostrata scammonii</i>]	MRK	S--YSGVARTSS	-GR	L R K L G D P T G P
XP_026936614.1 [<i>Lagenorhynchus obliquidens</i>]	MRK	S--YSGVMRTS	G-GR	L R K L G D P T G P
XP_024616632.1 [<i>Neophocaena asiaeorientalis asiaeorientalis</i>]	MRK	S--YSGVTRTSS	G-GR	L R K L G D P T G P
XP_010949017.1 [<i>Camelus bactrianus</i>]	MKK	S--YSGVARTSS	-GR	L R K L G D P T G P
KAF6458727.1 [<i>Rousettus aegyptiacus</i>]	MKK	S--YSGVTRTSS	-GR	L R K L G D P T G P
KAF6299646.1 [<i>Rhinolophus ferrumequinum</i>]	MKK	S--YTG VTRTSS	-GR	L R K L G D P T G P
KAF6417104.1 [<i>Molossus molossus</i>]	MKK	S--YSGVTRTSS	-GR	L R K L G D S T G P
XP_019499882.1 [<i>Hipposideros armiger</i>]	MKK	S--YTG VTRTSS	-GR	L R K L G D T T G P
XP_017919201.1 [<i>Capra hircus</i>]	MKK	S--YTG VTRTSS	-GR	L R K L G D S T G P
XP_004860849.1 [<i>Heterocephalus glaber</i>]	MKK	S--YSGVTRTAS	-GR	LRR LGDPTGP
XP_023507708.1 [<i>Heterocephalus glaber</i>]	MKK	S--YSGVTRTSS	-GR	LRR LGDLTGP
XP_015864047.1 [<i>Peromyscus maniculatus bairdii</i>]	MKK	T--NSGVARTSS	-GR	LRR LADPTGP
XP_021496060.1 [<i>Meriones unguiculatus</i>]	MKK	S--YSGVGR TSS	SGGR	LRR LADPTGP
XP_031210187.1 [<i>Mastomys coucha</i>]	MKK	S--YSGVTRTSS	-GR	LRR LADPTGP
XP_012968864.1 [<i>Mesocricetus auratus</i>]	MKK	S--YSGVTRTSS	-GR	LRR LADSTGP
VTJ77022.1 [<i>Marmota monax</i>]	MKK	S--YSGVTRTSS	-GR	LRR LGDPTGP
XP_010340668.1:1-25 [<i>Saimiri boliviensis boliviensis</i>]	MKK	S--YSGGTRTAS	-GR	LRR LGDPSGP
XP_030779009.1 [<i>Rhinopithecus roxellana</i>]	MKK	S--YSGGTRTSS	-GR	LRR LGDPSGP
XP_032097420.1 [<i>Sapajus apella</i>]	MKK	S--YSGGTRTSS	-GR	LRR LGDASGP
Sept9_i1 [<i>Homo sapiens</i>]	MKK	S--YSGGTRTSS	-GR	LRR LGDSSGP

Conservation



b

Consensus sequence



