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## **A role for Septin 2 in Drp1-mediated mitochondrial fission**

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36 **Abstract**

37

38 Mitochondria are essential eukaryotic organelles which often form intricate networks.  
39 The overall network morphology is determined by mitochondrial fusion and fission.  
40 Among the multiple mechanisms that appear to regulate mitochondrial fission, the ER  
41 and actin have recently been shown to play an important role by mediating  
42 mitochondrial constriction and promoting the action of a key fission factor, the  
43 dynamin-like protein Drp1. Here, we report that the cytoskeletal component Septin 2 is  
44 involved in Drp1-dependent mitochondrial fission in mammalian cells. Septin 2 localizes  
45 to a subset of mitochondrial constrictions and directly binds Drp1, as shown by  
46 immunoprecipitation of the endogenous proteins and by pulldown assays with  
47 recombinant proteins. Depletion of Septin 2 reduces Drp1 recruitment to mitochondria  
48 and results in hyperfused mitochondria and delayed FCCP-induced fission. Strikingly,  
49 septin depletion also affects mitochondrial morphology in *Caenorhabditis elegans*,  
50 strongly suggesting that the role of septins in mitochondrial dynamics is evolutionarily  
51 conserved.

52

## 53 **Introduction**

54

55 Mitochondria are highly dynamic eukaryotic organelles, which form an interconnected  
56 and dynamic network. Mitochondrial morphology and function are tightly interrelated.  
57 The dynamic behaviour of mitochondria relies partly on their movement along  
58 cytoskeletal tracks, including microtubules, intermediate filaments and actin (reviewed  
59 in [1]). In addition, mitochondrial network dynamics are determined by mitochondrial  
60 fusion and fission, to which the cytoskeleton also contributes. The molecular basis of  
61 mitochondrial dynamics is intensely studied from both the medical and the fundamental  
62 cell biology standpoints, as defects in this process can lead to mitochondrial dysfunction  
63 and to pathology [2-5]. The canonical mitochondrial fission mechanism involves the  
64 dynamin-like protein Drp1, which is recruited from the cytosol to mitochondria, where  
65 it oligomerizes to form spirals that constrict the mitochondrion [6]. Drp1 recruitment  
66 and oligomerization are highly regulated at the molecular level through  
67 posttranslational modifications of Drp1 [7] and through the presence of several Drp1  
68 receptors on the mitochondrial outer membrane [8-14]. Drp1-mediated fission is  
69 facilitated by ER-tubules that wrap around mitochondria and constrict them. An ER-  
70 localized formin, Inverted Formin 2 (INF2, [15]) and a mitochondria-localized Spire1  
71 isoform (Spire1C, [16]) cooperate to induce localized actin polymerization at the  
72 constriction sites [16]. In addition to this, several actin-binding proteins have been  
73 reported to participate in mitochondrial fission [17,18]. The motor protein Myosin 2  
74 (Myo2), proposed to provide actin with the necessary contractile force for  
75 mitochondrial fission ("mitokinesis" [18]), is known to not only interact with actin, but  
76 also with septins [19]. Septins are conserved eukaryotic GTP-binding proteins that are  
77 considered to be components of the cytoskeleton as they can form non-polar filaments  
78 (reviewed in [20,21].) Septins are highly expressed in interphase cells [22], suggesting  
79 that they play a role beyond their well-established contribution to cytokinesis [23,24].  
80 Indeed, recent studies indicate that septins are involved in a number of different cellular  
81 processes, such as ciliogenesis [25,26], axon guidance [27], phagocytosis [28] and serve  
82 to restrict bacterial actin-based motility [29] and protein diffusion both at the cell cortex  
83 [30], at the yeast bud neck [31] and at the ER [32]. Knockout of any of the 13  
84 mammalian septins leads to highly diverse phenotypes, further indicating that different

85 septins may perform different functions [21]. Mammalian septins are subdivided in 4  
86 groups (Septin 2, Septin 3, Septin 6, Septin 7) and form heteropolymeric complexes with  
87 the ubiquitous Septin 2 (Sept2) at the heart of the complexes [20]. Septins display a  
88 complex localisation pattern: long fibers occur under the nucleus and along actin stress  
89 fibers, while both long and short fibers are found throughout the cell, accumulating at  
90 the cell periphery in association with actin (reviewed in [20]). Furthermore, ring-like  
91 structures decorate the cytosol and subcortical regions [33].

92         In contrast to actin filaments and microtubules, septins are not known to interact  
93 extensively with mitochondria. To our knowledge, a single report indicated that septins  
94 colocalize with mitochondria in the ciliate *Tetrahymena thermophila* and septin  
95 depletion/overexpression grossly disrupted mitochondrial morphology in this organism  
96 [34]. Nevertheless, the mechanism through which ciliate septins act in mitochondrial  
97 dynamics has remained elusive. Concerning mammalian septins, knockout of the  
98 differentially expressed septin 4 (Sept4) in mice has been shown to result in sperm  
99 defects, including aberrant annulus and mitochondrial architecture [35]. Two Sept4  
100 splice isoforms have furthermore been found to localize to mitochondria and participate  
101 in apoptosis and neuronal development respectively [36,37]. To date, it is unclear  
102 whether any of the ubiquitously expressed septins is involved in mitochondrial  
103 dynamics of metazoan cells.

104         Here, we show that in mammalian cells Sept2 directly interacts with the  
105 mitochondrial fission protein Drp1 and is required for efficient localization of Drp1 at  
106 mitochondria, thus introducing septins as new players in mitochondrial dynamics.

107

## 108 **Results**

109

### 110 **Sept2 depletion induces mitochondrial elongation**

111 We assessed the role of septins in mitochondrial dynamics by silencing three members  
112 of the family (i.e. Sept2, Sept7 and Sept9) and analyzing mitochondrial morphology  
113 through indirect immunofluorescence (**Fig 1A**). Mitochondria were significantly  
114 elongated in Sept2 and Sept7 silenced cells compared to control cells, respectively by  
115 1.8-fold and 1.4-fold. In contrast, mitochondrial length did not significantly increase in  
116 Sept9-depleted cells (**Fig 1B**). Previous studies have shown that depletion of Sept7 co-  
117 depletes Sept2 [33,38,39], which could explain why depletion of either Sept2 or Sept7  
118 causes an increase in mitochondrial length. We therefore assessed the levels of Sept2  
119 upon Sept2, Sept7 and Sept9 depletion. In our hands, the depletion efficiency of Sept2  
120 reached almost 90%, while that of Sept7 reached 80% and resulted in a concomitant  
121 65% decrease in Sept2 levels, in agreement with previous reports [33,38,39]. Our very  
122 efficient Sept9 depletion (97%) did not significantly co-downregulate Sept2 (**expanded**  
123 **version Fig EV1A-D**). These findings are consistent with our observation that the  
124 depletion of Sept2 and Sept7, but not that of Sept9, affect mitochondrial length.

125 Given the strong mitochondrial phenotype obtained upon Sept2 depletion, we  
126 decided to focus our attention on Sept2 and its possible involvement in mitochondrial  
127 dynamics. To ensure that the observed mitochondrial elongation in Sept2 depleted cells  
128 is not due to an off-target effect, we confirmed the phenotype with different Sept2-  
129 targeting siRNA sequences (**expanded version Fig EV1E-F**) and in different cell types  
130 (HeLa, **Fig 1A** and U2OS, **Fig 2D**). Furthermore, the mitochondrial elongation phenotype  
131 of Sept2 silenced cells could be rescued through overexpression of siRNA-resistant  
132 Sept2 (**Fig 1C** and **1D**). Interestingly, Sept7 overexpression could also rescue the  
133 mitochondrial elongation phenotype induced by Sept2 siRNA, albeit less efficiently  
134 compared to the Sept2 siRNA-resistant construct, i.e. 54% rescue upon Sept7  
135 overexpression compared to 70% rescue for the overexpression of siRNA resistant  
136 Sept2 (**Fig 1D**), further suggesting that both proteins play a role in mitochondrial (see  
137 discussion).

138 We next asked whether increasing the amount of Sept2 would induce  
139 mitochondrial fission. Similar to Drp1 overexpression [40], overexpression of HA-tagged  
140 Sept2 did not substantially induce mitochondrial fragmentation (our unpublished

141 results), consistent with the notion that mitochondrial fission is a well-controlled  
142 multifactorial process, with multiple rate-limiting factors.

143         Since septins have been implicated in ER polarization in yeast [32], we sought to  
144 determine whether Sept2 depletion would affect the morphology of the ER or of other  
145 organelles, such as the Golgi apparatus and peroxisomes. The ER and Golgi morphology  
146 was not visibly altered in Sept2 depleted cells (**Fig 1E**). Similar mean areas were  
147 detected for the Golgi and the ER-mitochondria overlap in mock and siRNA-treated cells  
148 (**Fig 1F and 1G**). Moreover, live cell imaging showed that the ER marked mitochondrial  
149 fission sites to the same extent in control and in Sept2 siRNA-treated cells (**expanded**  
150 **view Fig EV1G**). Likewise, the number and size of peroxisomes were not significantly  
151 changed in Sept2-depleted cells compared to mock-treated cells (**Fig 1E and 1H**),  
152 suggesting that, although mitochondria and peroxisomes share components of the  
153 fission machinery [13,41{Koch, 2005 #3123}], Sept2 acts specifically in mitochondrial  
154 dynamics.

155

### 156 **Sept2 does not affect fusion but controls mitochondrial fission**

157 The mitochondrial hyperfusion phenotype could result from an increase in fusion  
158 activity or a decrease in fission. We thus tested whether Sept2 depletion would affect  
159 global levels of the key mitochondrial dynamics proteins Mfn1/2 and Drp1. Total levels  
160 of Mfn1/2 and Drp1 were not affected by Sept2 siRNA (**expanded view Fig EV2A and**  
161 **EV2B**). Live cell imaging revealed that Sept2 depleted cells displayed an increase in  
162 mitochondrial motility (**expanded view Fig EV2C and EV2D**), which may allow more  
163 frequent encounters between mitochondria and thus higher fusion rates [15]. We  
164 therefore tested whether Sept2 stimulates mitochondrial fusion activity. To measure the  
165 mitochondrial fusion rate, we employed an established assay, in which mitochondrial  
166 photoactivatable GFP (mito PA-GFP) is activated in a small region of interest, whose  
167 decay in fluorescence is followed over time to estimate the number of mitochondrial  
168 fusion events (i.e. the fusion rate), from which the fluorescence decay depends [42].  
169 Using this assay, we could not detect any significant increase in mitochondrial fusion  
170 when comparing Sept2 silenced cells with mock-treated cells (**Fig 2A and 2B**).

171         Given that Sept2 depletion resulted in a elongated mitochondria without  
172 increasing mitochondrial fusion, we analyzed whether Sept2 functionally contributes to  
173 mitochondrial fission by measuring fission rates in mock-treated and Sept2-depleted

174 cells. Sept2 depleted cells display decreased fission rates (mean  $14.8 \pm 5.7$ ) compared to  
175 mock treated cells ( $21.97 \pm 10.8$ , **Fig 2C**). Sept2 depleted cells also differed with respect  
176 to FCCP-induced mitochondrial fission and looping [43], which appeared delayed in  
177 Sept2 depleted cells compared to cells treated with control siRNA (**Fig 2D** and **2E**).  
178 Given that FCCP-induced mitochondrial fission is Drp1 dependent [44-49], this suggests  
179 that Sept2 may play a role in Drp1-dependent mitochondrial fission.

180

### 181 **Sept2 localizes at mitochondrial fission sites**

182 We reasoned that, given the effect of Sept2 depletion on mitochondrial morphology and  
183 its implication in FCCP-induced mitochondrial fission, a fraction of Sept2 would localize  
184 at mitochondria to regulate mitochondrial dynamics. We thus inspected the localisation  
185 of endogenous Sept2 by confocal microscopy and found Sept2 structures at  
186 mitochondria in HeLa (**Fig 3A** and **3B**) and U2OS cells (**Fig 3C**). In addition, we detected  
187 Sept2 in a crude mitochondrial fraction (**expanded view Fig EV3A**). 3D image  
188 reconstruction showed that endogenous Sept2 localizes above and around constricted  
189 mitochondria (**Fig 3B**, white arrowheads). Notably, we also found constrictions that  
190 were not marked by Sept2 (**Fig 3B**, yellow arrowhead). This might be due to 1) a  
191 transient association of Sept2 at mitochondrial fission sites ; 2) participation of Sept2 to  
192 only a subset of fission events, similar to what has been suggested for Myo2 and INF2  
193 [15,18] ; or 3) that other mechanisms ensure mitochondrial constriction in parallel. To  
194 achieve a clearer view of Sept2 structures at mitochondria we turned to superresolution  
195 imaging, using two complementary superresolution approaches : BioAxial's conical  
196 diffraction (CoDiM) based super-resolution method (**Fig 3C**) [50] and Structured  
197 Illumination Microscopy (SIM) (**expanded view Fig EV3B**). Where mitochondria were  
198 not obscured by strong cytosolic Sept2 staining, we detected Sept2 on 36% of  
199 constriction sites (n=209 constriction sites), which short Sept2 structures often  
200 traversing the mitochondria in a perpendicular way (**Fig 3C**).

201 We then monitored the dynamics of Sept2-YFP localization on mitochondria and  
202 in agreement with immunofluorescence analysis found Sept2 associated with  
203 mitochondria at prospective fission sites. We detected both discrete Sept2 puncta as  
204 well as Sept2-enriched halos on prospective mitochondrial fission sites (**Fig 3D**).  
205 Collectively, these results indicate that Sept2 localizes to mitochondria and is involved in  
206 mitochondrial fission.



207

## 208 **Sept2 interacts directly with Drp1**

209 We then sought to determine how Sept2 would act on mitochondrial fission. Given that  
210 Drp1-dependent mitochondrial fission is delayed in Sept2-depleted cells (**Fig 2D** and  
211 **2E**), we hypothesized that Sept2 might interact with Drp1. Consistent with this  
212 hypothesis, immunoprecipitation of endogenous Sept2 resulted in co-precipitation of  
213 endogenous Drp1 from cell lysates of several different human cell types (HeLa, HEK293  
214 and U2OS), indicating *in vivo* association of Drp1 and Sept2 (**Fig 4A, expanded view Fig**  
215 **EV3C and EV3D**). In contrast, actin or the mitochondrial outer membrane protein  
216 Tom20 did not co-precipitate in HeLa cells (**Fig 4A**), nor did INF2 in U2OS cells  
217 (**expanded view Fig EV3D**). To further verify the specificity of the Sept2-Drp1  
218 interaction, we immunoprecipitated an unrelated cytosolic protein (PI4KII $\alpha$ ) and did  
219 not recover any Drp1 (**expanded view Fig EV3E**). The interaction between Sept2 and  
220 Drp1 is likely to be direct because recombinant Sept2 interacted with Drp1 in GST  
221 pulldown experiments (**Fig 4B**). To investigate whether the Drp1-Sept2 interaction  
222 occurred preferentially in the cytoplasm or on mitochondria, we treated cells with CCCP  
223 to increase Drp1 localization to mitochondria. Treatment with CCCP caused a twofold  
224 increase in Drp1 binding to Sept2, suggesting that formation of the Sept2 - Drp1 complex  
225 is stimulated at mitochondria (**Fig 4C and 4D**). Immunofluorescence and live cell  
226 imaging confirmed that Sept2 and Drp1 can be found together at mitochondria (**Fig 4E**)  
227 and at mitochondrial fission sites (**Fig 4F**). We noticed that the two proteins do not  
228 colocalize at all prospective fission sites, suggesting that either the Sept2-Drp1 complex  
229 forms very transiently, or that Sept2 is below the detection level at these sites, or that  
230 Drp1 is recruited to mitochondria through several independent pathways. We next  
231 asked whether the interaction between Sept2 and Drp1 was sensitive to the activation  
232 state of Drp1. We thus treated cells with Mdivi-1, which inhibits self-assembly and  
233 GTPase activity of Drp1, resulting in increased interconnectivity of the mitochondrial  
234 network ([51], **expanded view Fig EV4F**) without depolymerizing Sept2 filaments (our  
235 unpublished results), and then performed Sept2 immunoprecipitation. Mdivi-1  
236 treatment decreased the association between Sept2 and Drp1 by almost 50% in intact  
237 cells (**Fig 4G and 4H**), and partially decreased the Sept2-Drp1 interaction in an *in vitro*  
238 pulldown assay (**expanded view Fig EV3G**).

239 Together, these data establish that Sept2 directly interacts with Drp1 *in vivo* and  
240 *in vitro* and suggest that the activation state of Drp1, its assembly or the molecular  
241 environment on the mitochondrial outer membrane may stabilize the Sept2-Drp1  
242 complex.

243

#### 244 **Mitochondrial localization of Drp1 depends on Sept2**

245 Since Sept2 interacts with Drp1, we analyzed the effect of Sept2 depletion on the  
246 mitochondrial localization of Drp1 complexes. Immunofluorescence analysis showed  
247 that the amount of mitochondria-associated Drp1 significantly decreased in Sept2  
248 silenced cells (67%±15.1) compared to mock-treated cells (**Fig 5A** and **5B**). In  
249 concomitance, the average distance between Drp1 clusters increased by a factor of 1.6 in  
250 Sept2-silenced cells (**Fig 5C**), indicating a decrease in Drp1 cluster density along  
251 mitochondria. In addition, western blotting analysis of mitochondria isolated from cells  
252 treated with control siRNA or with Sept2 siRNA confirmed a decrease in the amount of  
253 mitochondria-associated Drp1 (**Fig 5D**). These data suggest that Sept2 acts upstream of  
254 Drp1, similar to what has been recently shown for INF2 and Myo2 [15,18]. In agreement  
255 with this finding, Drp1 depletion did not affect Sept2 levels or subcellular distribution,  
256 as assessed by immunofluorescence (our unpublished results). Altogether, our  
257 observations revealed that Sept2 participates to the localization of Drp1 complexes on  
258 mitochondria.

259

#### 260 **Mitochondria-associated actomyosin is not perturbed upon Sept2 depletion**

261 Actin and Myosin 2 (Myo2) have been recently proposed to act together in  
262 mitochondrial precontraction, favouring Drp1 accumulation in order to accomplish  
263 fission [18]. Myo2 had also been shown previously to directly bind Sept2 [19]. This  
264 binding was required for phosphorylation-dependent activation of Myo2, which played  
265 an important role in stress fiber maintenance and during cytokinesis [19]. However,  
266 whether the Sept2-Myo2 interaction can also occur at mitochondria and/or have a  
267 functional role in mitochondrial dynamics is unknown to date. Therefore, we explored  
268 whether Sept2 depletion would alter Myo2 localization at mitochondria or decrease its  
269 activation. We neither detected changes in total Myo2A or Myo2B levels (**expanded**  
270 **view Fig EV4A and B**) nor a decrease in the levels of phosphorylated regulatory myosin  
271 light chain (P-MLC and PP-MLC, **expanded view Fig EV4C-F**), which determines the

272 actin-binding properties of Myo2 in Sept2 silenced cells [52]. We also failed to detect a  
273 significant change in mitochondria-associated P-MLC by immunolocalization (**Fig 6A**  
274 and **6B**). We thus concluded that the mitochondrial elongation observed in Sept2  
275 depleted cells is not due to perturbation of Myo2 levels or activity.

276 Notably, P-MLC has been shown to localize to mitochondria in an actin-  
277 dependent manner [18]. The lack of P-MLC redistribution in Sept2-depleted cells  
278 suggests that the interaction of actin and mitochondria is not perturbed in this context.  
279 In agreement with this observation, we found that Sept2 depletion did not interfere with  
280 the induction of actin recruitment to mitochondria upon FCCP treatment [17] (**Fig 6C**).  
281 In addition, we examined whether Sept2 depletion would affect the mitochondrial  
282 localization of the actin binding proteins Arp3, cofilin and cortactin, which were recently  
283 shown to regulate mitochondrial morphology [17]. Immunofluorescence and cell  
284 fractionation experiments showed that the mitochondrial association of Arp3, cofilin  
285 and cortactin was not affected in Sept2-depleted cells (**Fig 6D and E**).

286 We next tested whether the interaction between Sept2 and Drp1 requires actin  
287 polymerization by treating cells with cytochalasin D prior to immunoprecipitation and  
288 found that the Sept2-Drp1 interaction was not affected (**Fig 6F**). Together, these results  
289 indicate that Sept2 is not involved in the recruitment of actin and Myo2 at mitochondria  
290 and that the interaction between Sept2 and Drp1 is independent of actin dynamics.

291

### 292 **Septin contribution to mitochondrial dynamics is conserved in *C.elegans***

293 To understand whether the septin contribution to mitochondrial dynamics is conserved  
294 among metazoa, we turned to *C. elegans*, which possesses only two septins, *UNC-59* and  
295 *UNC-61*. We inspected mitochondrial morphology in body wall muscle cells, a well-  
296 established model to assess mitochondrial dynamics phenotypes [53-55]. Knockdown of  
297 either *UNC-59* or *UNC-61* or both by siRNA dramatically affected mitochondrial  
298 morphology, causing an increase in mitochondrial branching and length (**Fig 7A, 7B and**  
299 **expanded version Fig EV5A and EV5B**) that is reminiscent of fission protein  
300 knockdown [54-56]. We then assessed whether combining the depletion of septins with  
301 the depletion of Drp1 would result in a further increase in mitochondrial length, which  
302 would indicate that the two proteins act on mitochondria through different pathways.  
303 We chose to focus on *UNC-61*, whose depletion results in a stronger mitochondrial  
304 phenotype compared to *UNC-59*, and co-silenced it with *DRP-1*. The strong

305 mitochondrial phenotype of *DRP-1*(RNAi) worms was not exacerbated when *UNC-61*  
306 was co-silenced, suggesting that the phenotypes induced by *DRP-1* and *UNC-61*  
307 knockdown are not additive (**Fig 7C**). These data are consistent with the phenotype  
308 observed in mammalian cells and suggest that the function of septins in mitochondrial  
309 dynamics is conserved in *C.elegans*.  
310

## 311 Discussion

312

313 Mitochondrial fission is a highly regulated process and recent progress has highlighted  
314 novel roles for several cellular components in this process, such as the ER, actin and  
315 multiple actin-binding proteins [15-18,43,57]. Here, we add a new player to this  
316 complex picture and report on the role of septins, a component of the cytoskeleton, in  
317 mitochondrial dynamics. We describe that septin expression is important for  
318 mitochondrial morphology in human cell lines and in the nematode *C.elegans*. In human  
319 cells, depletion of three ubiquitously expressed septins (Sept2, Sept7 and Sept9) showed  
320 that both Sept2 and Sept7 are important for mitochondrial fission, as their depletion  
321 induces significant mitochondrial elongation. In line with previous results [33,38,39], we  
322 found that that depletion of Sept7 partially co-depletes Sept2. The remaining Sept2 may  
323 account for the decreased penetrance of the mitochondrial phenotype in Sept7-silenced  
324 cells compared to Sept2-silenced cells. Furthermore, the small levels of Sept2 remaining  
325 after Sept2 siRNA may serve as seeds that allow Sept7 to partially rescue the  
326 mitochondrial phenotype when overexpressed in Sept2 silenced cells. In contrast, Sept9  
327 depletion did not induce mitochondrial elongation, but appeared to increase  
328 mitochondrial interconnectivity, similar to nocodazole treatment [43,58]. We speculate  
329 that the differences between Sept2/7 and Sept9 may be related to the position of Sept9  
330 in the septin heterocomplex [59] or to the presence of Sept2-containing complexes in  
331 Sept9 depleted cells that still act on mitochondria. Such Sept2-containing complexes  
332 were previously shown to form short filaments in Sept9 depleted cells [59]. In  
333 agreement with this, we did not detect Sept2 codepletion in Sept9 silenced cells.

334 In our hands, Sept2 presented the most dramatic mitochondrial phenotype,  
335 raising the question of how Sept2 mechanistically impacts mitochondrial dynamics. We  
336 propose that Sept2 plays a role in Drp1-dependent mitochondrial fission. This notion is  
337 supported by several observations: first, mitochondrial association of Drp1 is impaired  
338 in Sept2-depleted cells. Second, FCCP-induced mitochondrial fission, which is Drp1-  
339 dependent, is delayed in Sept2 depleted cells. Lastly, Sept2 interacts directly with Drp1.  
340 An indirect confirmation of our results is the recent finding that Sept5 and Sept11 bind  
341 dynamin in neuronal cells [60]. The high homology of dynamin and Drp1 suggests that  
342 their interaction with septins may be ancestral. The interaction between Sept2 and Drp1  
343 appears to require Drp1 assembly or activation, as it is sensitive to Mdivi-1, which has

344 been shown to shown to inhibit the Drp1 assembly-dependent GTPase activity [51]. In  
345 our hands, the effect of Mdivi-1 was stronger *in vivo* than *in vitro*, indicating that the  
346 molecular environment on the mitochondrial outer membrane may stabilize the Sept2-  
347 Drp1 complex.

348 In contrast, Sept2-mediated GTP hydrolysis may be dispensable for this interaction,  
349 as overexpression of a GTPase-deficient point mutant (Sept2-T78G) did not affect  
350 mitochondrial morphology (our unpublished results), consistent with the very low rate  
351 of GTP hydrolysis that has been observed by others in septin heterooligomers [61,62]  
352 and which appears to be due to the low solvent accessibility of the nucleotide-binding  
353 pocket [63].

354 We propose alternative but not mutually exclusive scenarios that could account for  
355 the role of Sept2 in Drp1-dependent mitochondrial fission. Sept2 could promote Drp1  
356 recruitment by contributing to mitochondrial constriction. Alternatively, Sept2 might  
357 stabilize productive fission complexes or act as a scaffold that contributes to Drp1  
358 recruitment or retention on mitochondria, as has been proposed for actin [43,57].  
359 Notably, despite the fact that septins can use actin as an assembly template and partially  
360 colocalize with actin [33], we suspect that Sept2 does not play a substantial role in  
361 actomyosin driven mitochondrial fission [18]. In agreement with this hypothesis, actin  
362 and INF2 were not detected in Sept2 immunoprecipitations and cytochalasin D  
363 treatment had no effect on the Sept2–Drp1 interaction, the latter suggesting that it does  
364 not require actin polymerization. Furthermore, neither the localization of P-MLC on  
365 mitochondria, which relies on actin, is affected in Sept2-depleted cells, nor are the total  
366 levels of Myo2A, Myo2B, P-MLC or PP-MLC. P-MLC and PP-MLC were reported to  
367 decrease in previous Sept2-depletion experiments [19], a variation that may be  
368 explained by differences in the employed cell types (CHO-K1 versus HeLa and U2OS) or  
369 silencing techniques (shRNA versus siRNA). Interestingly, Li et al reported increased  
370 Drp1 recruitment to mitochondria upon depletion of the actin regulating proteins Arp3,  
371 cortactin and cofilin [17], while we observe a decreased localisation of Drp1 to  
372 mitochondria in Sept2-depleted cells and no substantial difference in mitochondrial  
373 localization of Arp3, cortactin and cofilin. Consistent with our findings, FCCP – induced  
374 recruitment of actin to mitochondria was also preserved in Sept2-depleted cells.

375 Our data support the view that multiple mechanisms regulate mitochondrial  
376 fission. On the one hand, accumulating evidence suggests the presence of Drp1-

377 independent fission mechanisms [48,64]. On the other hand Drp1 is regulated in a highly  
378 complex manner, i.e. through several Drp1 receptors on mitochondria, through  
379 posttranslational regulation of Drp1 itself, and through the regulated recruitment of  
380 Drp1 to mitochondria by its receptors Fis1, Mid49/51 and Mff [11,12];[10,13,14,65,66].  
381 The recruitment and activity of Drp1 on mitochondria is furthermore regulated by  
382 cellular structures such the ER [67] and actin [15,18,43,57], as well as septins, which  
383 appear to function in parallel to the ER and actin. Hence, the mitochondrial fission  
384 process appears to be regulated in complex and at least partially redundant ways. Such  
385 redundancy is frequently observed in biological systems and is thought to contribute to  
386 their robustness. Although not the only means to recruit Drp1 to mitochondria, Sept2  
387 has in contrast to the ER and actin the unique ability to directly bind Drp1. This could  
388 allow Sept2 to act as a scaffold that would promote functional interactions with other  
389 proteins on mitochondria, similar to the function that septins have already been shown  
390 to fulfil in other contexts such as cytokinesis [19]. Our findings thus unveil a new facet of  
391 Drp1 regulation and expand the role of the cytoskeleton in mitochondrial fission to  
392 septin proteins.

393

394

## 395 **Materials and Methods**

396

### 397 **Cell culture and transfection**

398 HeLa and U2OS cells were obtained from ATCC and cultured under standard conditions;  
399 media and additives were from Invitrogen. U2OS-GFPsec61 stable cells were described  
400 previously (Shibata 2008), as well as Drp1 <sup>-/-</sup> cells [45]. All cells were tested for  
401 mycoplasma (negative). siRNAs were transfected for 72h at a final concentration of 12  
402 nM with Lipofectamine RNAiMax according to the manufacturer's instructions. Septin  
403 siRNA sequences and providers are detailed in supplementary table 1 ; siRNA#1 and  
404 siRNA#2 were employed for Fig 1 and Fig EV1A and B, while siRNA#1 is shown in  
405 subsequent experiments. For rescue experiments, HeLa cells were transfected with  
406 Sept2 siRNA (siRNA #1) for 48h and then transfected for a further 24 h (using Fugene)  
407 with a Sept2-HA plasmid carrying silent mutations in the siRNA seed region (Sept2-HA  
408 mut, see Supplementary Data table 1) or its GTPase deficient derivative (carrying the  
409 T78G mutation [63]) or Sept7-HA.

410

### 411 **Reagents**

412 Plasmids, chemicals and recombinant proteins: Sept2-HA template for cloning of siRNA-  
413 resistant Sept2-HA was provided by David Ribet. See supplementary table 2 for  
414 construct and primer information. PA-GFP and Sept2-YFP were described previously  
415 [42,68]. Orange and Deep Red Mitotracker, 100nm tetraspeck beads AlexaFluor-labeled  
416 Phalloidin and secondary antibodies were purchased from Invitrogen. Mdivi-1 was  
417 obtained from Enzo Life Sciences, all other chemicals were obtained from Sigma.  
418 Complete mini EDTA-free protease inhibitor and PhoStop phosphatase inhibitor tablets  
419 were from Roche. Reduced glutathione was obtained from Sigma, glutathione sepharose  
420 beads and Protein A were from GE Healthcare. Recombinant purified GST and Drp1-GST  
421 were prepared as described previously [69] and Sept2 was obtained from Cusabio (CSB-  
422 EP617994HU).

423 Antibodies: Antibody sources are detailed in table 3. All antibodies were used according  
424 to the manufacturer's instructions unless otherwise stated.

425

### 426 **Cell fractionation, immunoprecipitations and pulldown**



427 Crude mitochondrial extracts were obtained through cell fractionation according to [70]  
428 Immunoprecipitation was performed as described [29] with modifications. Briefly, HeLa  
429 cells were washed twice in phosphate-buffered saline (PBS) and lysed for 30 min with 1  
430 ml lysis buffer/10cm dish (20 mM Tris, pH 8.0, 150 mM NaCl, 10% glycerol)  
431 supplemented with 1% Igepal, protease and phosphatase inhibitors. Lysis and all  
432 subsequent steps were performed at 4 °C. The lysate was clarified (13000xg, 10 min)  
433 and the protein concentration of the supernatant was determined by Bradford assay  
434 (Pierce). 1 mg of lysate was incubated overnight with 2 µg of anti Sept2 antibody. 30 µl  
435 of protein A Sepharose beads were added for 1 h and the immune complexes were  
436 retrieved by centrifugation (500x g, 5 min). After four washes with lysis buffer, bound  
437 protein was eluted from the beads by boiling for 10 min in 30 µl Laemmli buffer. The  
438 eluate was analyzed by gradient SDS-PAGE (Biorad), and subjected to western blotting  
439 via wet transfer to 0.45µm nitrocellulose membrane (Millipore). Ten µg total lysate were  
440 loaded (corresponding to 1/100) for the input, 70-90% immunoprecipitated material to  
441 reveal interactors such as Drp1, or 5% immunoprecipitated material to reveal  
442 immunoprecipitated Sept2. Sept2 immunoprecipitations from drug- treated cells were  
443 performed as above except that cells were either mock treated (DMSO or ethanol,  
444 respectively), or treated with either 50 µM Mdivi-1 for 1 h (Mdivi-1 was also included in  
445 the lysis buffer), or 2 µM CCCP for 30 min in MEM without serum, or 2 µM cytochalasinD  
446 for 30 min in MEM before cell lysis.

447 GST pull-down: All steps were performed at 4 °C. Equimolar concentrations of GST, GST-  
448 Drp1 and Sept2 (500 nM) were incubated overnight in GST incubation buffer (20 mM  
449 Tris, pH 8.0, 1mM EDTA, 0,2% Igepal, 100 mM KCl). Thirty µl of protein A Sepharose  
450 beads were added for 1 h; beads were then recovered by centrifugation (500xg, 5 min)  
451 and washed four times in GST incubation buffer. Bound protein was eluted from  
452 glutathione Sepharose beads with GST elution buffer (100 mM Tris, pH 8.0, 20 mM  
453 glutathione, 5 mM dithiothreitol). Eluates were analyzed as described above. To test the  
454 effect of Mdivi-1 on the Drp1-Sept2 interaction, Drp1-GST was preincubated with Mdivi-  
455 1 for 30 min at RT before addition of recombinant Sept2, followed by the pull-down  
456 procedure described above.

457

458 **Imaging and Image analysis**

459 Immunofluorescence and live cell imaging were performed essentially as described in  
460 [48,71]. For superresolution microscopy, cells were seeded onto high precision  
461 coverslips (Marienfeld) and after immunofluorescence samples were mounted in  
462 Slowfade Gold (Invitrogen) or Fluoromount G. Super-resolution structured illumination  
463 (SR-SIM), providing an expected resolution of about 140 nm, was performed on a Zeiss  
464 LSM780 Elyra PS1 (Carl Zeiss, Germany) using 63x/1.4 oil Plan Apo objective. Three  
465 angles of the excitation grid with five phases each were acquired for each channel and  
466 each z-plane. SIM images were processed with ZEN software for brightness/contrast  
467 adjustment. Images were aligned using 100 nm tetraspeck beads embedded in the  
468 sample. The confocal image on Fig EV3B was acquired using the same equipment as the  
469 SR-SIM and the same pixel size to allow comparison. Huygens professional software was  
470 used for deconvolution and brightness/contrast in image S3B. CoDIM imaging was  
471 performed on a Bioaxial CoDiM 100 system equipped with 488, 561 and 640 lasers and  
472 decreases the resolution limit to about 80-100 nm. Imaris 6.4.1 (Bitplane) was used for  
473 3D reconstructions.

474 Image analysis was performed with ImageJ unless otherwise stated. For  
475 morphometric analysis on fixed cells, mitochondrial length was assessed in well-  
476 resolved mitochondria only; fissions were counted manually in live cell imaging  
477 experiments and displayed according to [57]. To calculate the percentage of rescue, the  
478 difference in mitochondrial length between Sept2-siRNA and Sept2-rescue or Sept7-  
479 rescue is referred to the difference between Sept2-depleted and mock-treated cells (set  
480 to 100%). To quantify FCCP-induced mitochondrial fission and looping, mitochondrial  
481 elongation (long/short axis) was measured with the open-source software Icy [72]  
482 (protocol *Mitochondria Elongation*) after binarization of mitochondria. The first  
483 timepoint (t<sub>0</sub>) was set to 100% and the following timepoints were normalized to t<sub>0</sub>.  
484 Mitochondrial constrictions were determined through visual inspection of the  
485 mitochondrial channel and then septin presence was assessed at these sites. Total  
486 mitochondrial motility was determined in ImageJ according to [73]. Peroxisomes were  
487 counted automatically with ImageJ particle analyzer. The percentage of ER crossing  
488 mitochondria was assessed after thresholding and binarization of the ER and  
489 mitochondrial signal according to [67]. Similarly, mitochondrial Drp1 was obtained by  
490 combining total Drp1 signal with a mitochondrial mask created after thresholding and  
491 binarization of the mitochondrial signal. Colocalization of P-MLC was assessed similarly

492 and verified using the plugin *Colocalization Studio* (Plugin ID: ICY-H9X6X2) [74] in Icy.  
493 Western blot quantification involved densitometric analysis of single bands through  
494 ImageJ. At least two western blots from independent experiments were quantified.

495

#### 496 **Statistical analysis**

497 Results are expressed as means of at least two independent experiments, error bars  
498 represent the standard error of the mean. For multiple comparisons, data were first  
499 analyzed by one-way ANOVA on BiostaTGV  
500 (<http://marne.u707.jussieu.fr/biostatgv/?module=tests>), followed by pairwise  
501 comparisons with unpaired two-tailed Student's t-test on Excel (Microsoft) or Prism  
502 (Graphpad). Significance is indicated as  $p < 0.05$  (\*),  $p < 0.01$  (\*\*) and  $p < 0.005$  (\*\*\*), ns for  
503  $p > 0.05$ .

504

#### 505 ***C. elegans* work**

506 *Caenorhabditis elegans* was cultured and maintained as described previously [75] at  
507 20°C. The worm line carrying pMyo3::Tom70::GFP, rol-6 as extrachromosomal array  
508 was created previously [54]. RNAi was performed as described [76]. Plasmid L4440,  
509 containing *unc-59* (W09C5.2) or *unc-61* (Y50E8A.4) were retrieved from the Ahringer  
510 library [76] or the Vidal library [77], respectively. Both clones were sequenced to  
511 confirm their identity. For the double RNAi and the dilution controls, equal volumes of  
512 bacterial cultures were mixed, concentrated by centrifugation and spread on NGM plates  
513 containing 1.5 mM IPTG and 25 µg/ml carbenicillin. dsRNA production was induced at  
514 RT for 16 to 24 hours, then stored at 4°C for up to two weeks. TOM70::GFP worms were  
515 subjected to RNAi from L3/L4 stage for 4-5 days at 20°C. Offsprings were analyzed at  
516 young adult stage. For live imaging, adult hermaphrodites were mounted in M9  
517 containing 1 mM Levamisole (Sigma-Aldrich), using Vaseline® at the edges of the  
518 coverslip to function as a spacer. Worms and mounting medium were strictly kept at  
519 20°C until use. Images were taken at a Zeiss Axioplan 2 microscope equipped with a  
520 Zeiss AxioCam MRm camera (Carl Zeiss, Germany) and a Plan Neofluar 100×/NA1.30 oil  
521 objective.

522

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539

## 540 **Author contributions**

541 AP and FS conceived, performed and interpreted the experiments, TNT and JKS  
542 performed and interpreted experiments, RP, SO, ASa and TL provided expertise, JCOM  
543 supervised TL, FS wrote the paper, AP, ASp and PC reviewed and edited the manuscript,  
544 PC supervised the project and acquired funding.

545

## 546 **Conflict of Interest**

547 The authors declare no conflict of interest.

548

549

550 **Figure legends**

551 **Figure 1: Sept2 depletion affects mitochondrial morphology.** (A) Mitochondrial  
552 morphology in HeLa cells depleted of Sept2, Sept7 and Sept9. Mitochondria were labeled  
553 with mitotracker (green). Scale bar: 10  $\mu$ m. The inset represents a twofold enlargement.  
554 (B) Quantification of mean mitochondrial length in mock treated cells and in cells  
555 depleted of Sept2, Sept7 and Sept9.  $n > 250$  individual mitochondria from three  
556 independent experiments. (C) Mitochondrial morphology in mock treated cells or HeLa  
557 cells depleted of Sept2 and then transfected with empty vector, siRNA-resistant HA-  
558 tagged Sept2 (Sept2-rescue) or Sept7 (Sept7-rescue). Mitochondria were labeled with  
559 mitotracker (shown in green), asterisks mark transfected cells. Scale bar: 10  $\mu$ m, insets  
560 are twofold enlargements. (D) Quantification of mean mitochondrial length in mock  
561 treated cells, in cells depleted of Sept2 or in cells depleted of Sept2 and transfected with  
562 siRNA-resistant Sept2 (Sept2-rescue) or Sept7 (Sept7-rescue).  $n > 200$  individual  
563 mitochondria from two independent experiments. (E) Mock treated or Sept2 depleted  
564 U2OS cells stained for Sept2 (green) and the Golgi apparatus (GM130, red), the ER  
565 (Sec61b-GFP, displayed in red) or peroxisomes (PMP70, red). (F) Quantification of the  
566 mean Golgi area ( $n = 40$  cells, three independent experiments) (G) Quantification  
567 showing the percentage of ER traversing well-resolved mitochondrial tubules ( $n = 15$   
568 cells from three independent experiments). (H) Quantification of average peroxisome  
569 counts per cell ( $n = 40$  cells from three independent experiments). Scale bar: 10  $\mu$ m,  
570 insets are enlarged twofold.

571  
572 **Figure 2: Mitochondrial dynamics in Sept2 depleted cells.** (A) Live cell imaging of  
573 mock treated or Sept2 depleted U2OS cells analyzed with the mito-PA-GFP  
574 mitochondrial fusion assay. Mito-PA-GFP is photoactivated in the indicated ROI and the  
575 decrease in fluorescence is followed in the same ROI, correcting for cell movement (B)  
576 Quantification of mitochondrial fusion rates in mock treated or Sept2 depleted cells  
577 expressed as a means of three independent experiments  $\pm$  SEM. (C) Quantification of  
578 fission rates in mock and Sept2 depleted cells.  $n = 116$  fission events for Sept2 siRNA,  
579  $n = 83$  fission events for mock, two independent experiments. (D) Live cell imaging of  
580 U2OS cells stably transfected with a GFP targeted to the mitochondrial outer membrane  
581 (OM-GFP), showing delayed FCCP-induced mitochondrial fission in Sept2 depleted cells

582 compared to mock treated cells. White arrowheads point at fission examples, yellow  
583 arrowheads to looping. Scale bar: 10  $\mu\text{m}$ , insets are enlarged twofold. (E) Quantification  
584 of mitochondrial elongation (long /short axis) in mock treated and FCCP treated U2OS  
585 cells showing increased elongation in Sept2 depleted cells compared to mock treated  
586 cells, mean of four independent experiments  $\pm$  SEM.

587

588 **Figure 3: Sept2 localizes to mitochondrial constrictions.** (A) Immunofluorescence  
589 analysis of endogenous Sept2 (green) and mitochondria (Tom20, red) in HeLa cells. The  
590 insets show a twofold enlargement with arrowheads highlighting the presence of small  
591 Sept2 structures on mitochondria. Scale bar 10  $\mu\text{m}$ . (B) 3D rendering of confocal  
592 sections showing Sept2 at mitochondrial constrictions (white arrowheads) and  
593 constricted mitochondria that are not marked by Sept2 (yellow arrowhead) Scale bar 2  
594  $\mu\text{m}$ . (C) CoDiM super-resolution imaging of endogenous Sept2. Arrowheads highlight the  
595 presence of Sept2 at mitochondrial constrictions. Insets show a threefold enlargement  
596 Scale bar 10  $\mu\text{m}$ . (D) Still images from a live cell imaging experiment showing Sept2  
597 (green) accumulation during two mitochondrial fission events (mitotracker orange,  
598 red), highlighted by yellow (first fission) or green arrowheads (second fission). Scale bar  
599 2  $\mu\text{m}$ .

600

601 **Figure 4: Sept2 interacts with Drp1.** (A) Western blot analysis of endogenous Sept2  
602 immunoprecipitation from HeLa cells showing that endogenous Drp1 specifically co-  
603 immunoprecipitates with Sept2. The asterisks mark the IgG heavy chains. (B) Elyra SIM  
604 image of U2OS cells stained for Sept2 (green), mitochondria (GFP targeted to the outer  
605 membrane (OM-GFP), shown in red), Drp1 (blue). Yellow arrows point at tetraspeck  
606 beads used for image alignment. Insets represent sevenfold enlargements. White arrows  
607 in the insets point to sites where Drp1 and Sept2 colocalize on mitochondria. Scale bar:  
608 10  $\mu\text{m}$ , insets are enlarged fourfold. (C) GST pulldown of recombinant Sept2 with GST-  
609 Drp1 or GST alone showing that Sept2 is able to interact directly with Drp1. (D) Sept2  
610 immunoprecipitation from mock or CCCP-treated HeLa cells probed for Drp1 and Sept2.  
611 (E) Quantification of two independent experiments showing stimulated Drp1 co-  
612 immunoprecipitation with Sept2 in FCCP-treated cells. (F) Sept2 immunoprecipitation  
613 from mock or Mdivi-1-treated HeLa cells probed for Drp1 and Sept2. (G) Quantification  
614 of two independent experiments showing decreased Drp1 co-immunoprecipitation with

615 Sept2 upon Mdivi-1 treatment. (H) Snapshots of a live cell imaging experiment in U2OS  
616 cells showing Sept2 (green, Sept2-YFP), Drp1 (red, Drp1-mCherry) and mitochondria  
617 (blue, mitotracker DeepRed) during mitochondrial fission. Merged and single channels  
618 show the dynamics of Sept2 and Drp1 during mitochondrial fission (yellow  
619 arrowheads). Scale bar: 2  $\mu\text{m}$ .

620

621 **Figure 5: Sept2 is required for efficient recruitment of Drp1 to mitochondria.** (A)  
622 Immunofluorescence of Sept2 (blue), mitochondria (Tom20, red) and Drp1 (green) in  
623 mock treated and Sept2 depleted cells. Scale bar: 10  $\mu\text{m}$ , insets are enlarged fourfold. (B)  
624 Quantification of mitochondria-associated Drp1 in mock treated and Sept2 depleted  
625 cells (mean of 3 independent experiments, n=44-51 cells) (C) Average distance between  
626 mitochondria-associated Drp1 oligomers (representative experiment with n>25  
627 individual mitochondria). (D) HeLa cytosol and crude mitochondrial fractions prepared  
628 from mock and Sept2 siRNA treated cells. The samples were analysed by western  
629 blotting with indicated antibodies, and show a decrease in mitochondria-associated  
630 Drp1 in Sept2 depleted cells.

631

632 **Figure 6: Sept2 does not affect mitochondrial Myo2 localization and does not**  
633 **require actin dynamics for interacting with Drp1.** (A) Immunofluorescence analysis  
634 of P-MLC (green) recruitment to mitochondria (red, Tom20) in mock treated and Sept2  
635 depleted HeLa cells. Insets show twofold enlargements. Scale bar: 10  $\mu\text{m}$ . (B)  
636 Colocalization of P-MLC with mitochondria was assessed with Icy software and showed  
637 no difference between mock and Sept2 depleted cells. (C) Mock treated or Sept2  
638 depleted Drp1  $-/-$  MEF cells were labeled with mitotracker orange (red), treated for the  
639 indicated amount of time with 2 $\mu\text{M}$  FCCP and stained for actin with phalloidin (green).  
640 Insets are enlarged twofold. (D) Immunofluorescence analysis of the actin binding  
641 proteins Arp3 and cofilin (green), colocalized with cytochrome c (red) and cortactin  
642 (green), colocalized with Hsp60 (red) in mock treated or Sept2 depleted Drp1  $-/-$  MEF  
643 cells. Scale bars: 10  $\mu\text{m}$  (E) Cytosol and crude mitochondria fractions were prepared  
644 from mock treated or Sept2 depleted Drp1  $-/-$  MEF cells and analyzed by western  
645 blotting with the indicated antibodies. (F) HeLa cells were mock treated or treated with  
646 cytochalasin D and subjected to Sept2 immunoprecipitation. Immunoprecipitates were

647 analyzed by western blot for Drp1 and Sept2, showing that Drp1 co-  
648 immunoprecipitation with Sept2 is not affected by cytochalasin D treatment.

649

650 **Fig. 7 Mitochondrial structure in muscle cells is affected upon knockdown of *UNC-***  
651 ***59* and *UNC-61*.**

652 (A) Live imaging of *C. elegans* expressing TOM70::GFP in body wall muscle cells.  
653 Depletion of the *UNC-59* or *UNC-61* led to an increase in mitochondrial network  
654 connectivity as shown by quantification in (B). Insets represent a twofold magnification.  
655 Scale bar: 10  $\mu$ m. (C) Live imaging of *C. elegans* body wall muscle cells expressing  
656 TOM70::GFP. Depletion of *DRP-1* or *UNC-61* caused an increase in mitochondrial  
657 elongation and network connectivity, which did not increase upon combination of *DRP-1*  
658 and *UNC-61*. Insets represent a twofold magnification. Scale bar: 10  $\mu$ m.

659



660 **Expanded View Figures**

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663 **Expanded View Figure legends**

664

665 **Figure EV1 : Western blot quantification of septin depletion. Sept2 depletion**  
666 **affects mitochondrial morphology but not ER-dependent mitochondrial fission.**

667 (A) Western blot showing siRNA mediated silencing of septins. Sept2 is most efficiently  
668 depleted upon Sept2 siRNA. Sept7 siRNA also codepletes Sept2, while Sept9 siRNA does  
669 not affect Sept2 levels. (B-D) Quantification of Sept2, Sept7 and Sept9 protein level after  
670 siRNAs treatment, mean of at least three independent experiments. The red line serves  
671 as a reference, and highlights the minimal residual amount of Sept2, obtained upon  
672 treatment with Sept2 siRNA. (E) Representative images of Sept2 depleted HeLa cells  
673 showing mitochondrial elongation induced by two different siRNAs. (F) Quantification of  
674 the percentage of cells showing mitochondrial hyperfusion after treatment with each  
675 Sept2-targeting siRNA, n>150 cells from two independent experiments. (G)  
676 Quantification of ER-marked mitochondrial fissions in control and Sept2 siRNA treated  
677 cells according to [67].

678 **Figure EV2 : Analysis of mitochondrial motility and fission/fusion protein levels**  
679 **upon Sept2 depletion**

680 (A) Total lysates of mock treated or Sept2 depleted cells were  
681 analyzed by western blot and probed for Mfn1, Mfn2 and Drp1. (B) Western blot  
682 quantification of total Mfn1, Mfn2 or Drp1 levels in mock treated and Sept2 depleted  
683 total cell lysates, mean of two independent experiments. (C) Mock treated cells and  
684 Sept2 depleted cells were labeled with mitotracker orange and imaged on a spinning  
685 disk microscope for 10 minutes. Ten snapshot images, taken every 15s, were overlaid  
686 and colour-coded to visualize mitochondrial motility, with immotile mitochondria  
687 appearing white. (D) Quantification of mitochondrial motility according to [73]; ten  
688 individual timepoints in two independent experiments expressed as a mean  $\pm$ SEM. Scale  
bar: 10  $\mu$ m.

689 **Figure EV3 : Sept2 localises to mitochondria and interacts with Drp1 in different**  
690 **cell types**

691 (A) Total HeLa cell lysate as well as crude mitochondrial fraction and  
692 postnuclear supernatant were first analysed for mitochondrial enrichment by western  
blotting with Drp1, tubulin and Tom20 antibodies and then equal amounts were

693 reloaded and probed for Sept2. (B) Confocal (left panel) and Elyra SIM (right panel)  
694 image of U2OS cells labelled with Tom20 (red) and Sept2 (green). Insets show fourfold  
695 enlargements. Arrowheads show small Sept2 fibers colocalizing with mitochondria.  
696 Scale bar: 10  $\mu$ m. (C-D) Co-immunoprecipitation of endogenous Drp1 with endogenous  
697 Sept2 in HEK293 (C) and U2OS (D) cells. (E) Immunoprecipitation of endogenous  
698 PI4KII $\alpha$  in HeLa cells shows no co-immunoprecipitation of Drp1. (F)  
699 Immunofluorescence analysis of HeLa cells treated for 1 h with Mdivi-1 and stained with  
700 Tom20 to reveal mitochondrial morphology. Mdivi-1 treatment increased mitochondrial  
701 interconnectivity as previously described [51]. (G) GST pulldown of recombinant Sept2  
702 with GST-Drp1 or GST alone in the presence or absence of 50 $\mu$ M Mdivi-1, which partially  
703 decreased Sept2-Drp1 interaction.

704 **Figure EV4: Sept2 depletion does not decrease the phosphorylation level of**  
705 **myosin light chain** (A) Total lysates of Sept2 depleted HeLa cells were analyzed by  
706 western blot for Myo2B; equal amounts were reloaded and probed for Myo2A.  
707 Quantifications are shown in (B). (C) Western blot analysis of phosphorylated  
708 regulatory myosin light chain (P-MLC, Ser19) in mock treated or Sept2 depleted  
709 samples. The same samples were reloaded and analyzed by western blot for  
710 bisphosphorylated regulatory myosin light chain (PP-MLC, Thr18/Ser19).  
711 Quantifications are shown in (D) and (E).

712 **Figure EV5: Mitochondrial structure in muscle cells is perturbed by knockdown of**  
713 **UNC-59 and UNC-61.**

714 Live imaging of *C. elegans* expressing TOM70::GFP in body wall muscle cells. Depletion of  
715 *UNC-59* and *UNC-61* led to an increase in mitochondrial network connectivity. Empty  
716 Vector containing feeding bacteria served as a control and were mixed in 1:1 in control  
717 double feeding experiments. For double feeding RNAi experiments, equal amounts of  
718 *unc-59* and *unc-61* dsRNA expressing bacteria were used. Scale bar: 10  $\mu$ m.

719 **Expanded view tables**

720

721 **Table EV1 : siRNA**

siRNA target	Catalog number/sequence	Provider/reference
mock	D-001210-01-20	Dharmacon
mock	AGGUAGUGUAAUCGCCUUG	MWG [48]
Sept2 siRNA #1	14709	Invitrogen [78]
Sept2 siRNA #2	4735	Dharmacon
Mouse Sept2	s9420	Invitrogen
Sept7		
Sept9	18228	Invitrogen

722

723

724 **Table EV2 : Plasmids**

Plasmid	Oligos	reference
Sept2 HA - rescue	cgtttatgaa <b>CGCGATT</b> CACAACAAGGTGaatattgtg (siRNA targeting sequence bold uppercase, silent mutations in red)	This study
Sept2 HA - dGTP	aaaaattgaaaga <b>GGT</b> gtccagattgaggc (T78G mutation bold uppercase)	This study
Sept7-HA		Ribet et al, in preparation
Sept2-YFP		[68]
OM-GFP		[48]
Drp1-mCherry		[67]

725

726

727 **Table EV3 : Antibodies**

Antibody	Catalog number	Provider
Tom20	Clone 29	BD Biosciences
Drp1	611112	BD Biosciences
Myo2A	3403	Cell Signaling
Myo2B	3404	Cell Signaling
P-MLC2 (S19)	3671	Cell Signaling
P-P-MLC2 (W18/S19)	3674	Cell Signaling
Sept2	A304-211A	Bethyl
Gm130	610823	BD Biosciences
PMP70	Clone 70-18	Sigma
tubulin	Clone B5.1.2	Sigma
GAPDH	Clone 6c5	Abcam
actin	A5441	Sigma
CoxIV	Clone 3E11	Cell Signaling
HA	Clone 6E2	Cell Signaling
Cofilin	ab42824	Abcam
Cortactin	05-180	Millipore
Arp3	in house production	[79]
PI4KII $\alpha$	in house production	[80]

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734

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