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Abstract

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

Introduction

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

Results

Sept2 depletion induces mitochondrial elongation

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

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

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

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

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

Sept2 does not affect fusion but controls mitochondrial fission

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

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

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

Sept2 localizes at mitochondrial fission sites

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

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

Sept2 interacts directly with Drp1

We then sought to determine how Sept2 would act on mitochondrial fission. Given that Drp1-dependent mitochondrial fission is delayed in Sept2-depleted cells (**Fig 2D** and **2E**), we hypothesized that Sept2 might interactact with Drp1. Consistent with this hypothesis, immunoprecipitation of endogenous Sept2 resulted in co-precipitation of endogenous Drp1 from cell lysates of several different human cell types (HeLa, HEK293 and U2OS), indicating *in vivo* association of Drp1 and Sept2 (**Fig 4A**, **expanded view Fig EV3C and EV3D**). In contrast, actin or the mitochondrial outer membrane protein Tom20 did not co-precipitate in HeLa cells (**Fig 4A**), nor did INF2 in U2OS cells (**expanded view Fig EV3D**), To further verify the specificity of the Sept2-Drp1 interaction, we immunoprecipitated an unrelated cytosolic protein (PI4KIIα) and did not recover any Drp1 (**expanded view Fig EV3E**). The interaction between Sept2 and Drp1 is likely to be direct because recombinant Sept2 interacted with Drp1 in GST pulldown experiments (**Fig 4B**). To investigate whether the Drp1-Sept2 interaction occured preferentially in the cytoplasm or on mitochondria, we treated cells with CCCP to increase Drp1 localization to mitochondria. Treatment with CCCP caused a twofold increase in Drp1 binding to Sept2, suggesting that formation of the Sept2 - Drp1 complex is stimulated at mitochondria (**Fig 4C** and **4D**). Immunofluorescence and live cell imaging confirmed that Sept2 and Drp1 can be found together at mitochondria (**Fig 4E**) and at mitochondrial fission sites (**Fig 4F**). We noticed that the two proteins do not colocalize at all prospective fission sites, suggesting that either the Sept2-Drp1 complex forms very transiently, or that Sept2 is below the detection level at these sites, or that Drp1 is recruited to mitochondria through several independent pathways. We next asked whether the interaction between Sept2 and Drp1 was sensitive to the activation state of Drp1. We thus treated cells with Mdivi-1, which inhibits self-assembly and GTPase activity of Drp1, resulting in increased interconnectivity of the mitochondrial network ([51], **expanded view Fig EV4F**) without depolymerizing Sept2 filaments (our unpublished results),l and then performed Sept2 immunoprecipitation. Mdivi-1 treatment decreased the association between Sept2 and Drp1 by almost 50% in intact cells (**Fig 4G** and **4H**), and partially decreased the Sept2-Drp1 interaction in an *in vitro* pulldown assay (**expanded view Fig EV3G**).

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

Mitochondrial localization of Drp1 depends on Sept2

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

Mitochondria-associated actomyosin is not perturbed upon Sept2 depletion

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

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

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

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

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

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

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

Discussion

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

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

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

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

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

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

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

Materials and Methods

Cell culture and transfection

HeLa and U2OS cells were obtained from ATCC and cultured under standard conditions; media and additives were from Invitrogen. U2OS-GFPSec61 stable cells were described previously (Shibata 2008), as well as Drp1 -/- cells [45]. All cells were tested for mycoplasma (negative). siRNAs were transfected for 72h at a final concentration of 12 nM with Lipofectamine RNAimax according to the manufacturer's instructions. Septin siRNA sequences and providers are detailed in supplementary table 1 ; siRNA#1 and siRNA#2 were employed for Fig 1 and Fig EV1A and B, while siRNA#1 is shown in subsequent experiments. For rescue experiments, HeLa cells were transfected with Sept2 siRNA (siRNA #1) for 48h and then transfected for a further 24 h (using Fugene) with a Sept2-HA plasmid carrying silent mutantions in the siRNA seed region (Sept2-HA mut, see Supplementary Data table 1) or its GTPase deficient derivative (carrying the T78G mutation [63]) or Sept7-HA.

Reagents

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

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

Cell fractionation, immunoprecipitations and pulldown

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

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

Imaging and Image analysis

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

Image analysis was performed with ImageJ unless otherwise stated. For morphometric analysis on fixed cells, mitochondrial length was assessed in well-resolved mitochondria only; fissions were counted manually in live cell imaging experiments and displayed according to [57]. To calculate the percentage of rescue, the difference in mitochondrial length between Sept2-siRNA and Sept2-rescue or Sept7-rescue is referred to the difference between Sept2-depleted and mock-treated cells (set to 100%). To quantify FCCP-induced mitochondrial fission and looping, mitochondrial elongation (long/short axis) was measured with the open-source software Icy [72] (protocol *Mitochondria Elongation*) after binarization of mitochondria. The first timepoint (t0) was set to 100% and the following timepoints were normalized to t0. Mitochondrial constrictions were determined through visual inspection of the mitochondrial channel and then septin presence was assessed at these sites. Total mitochondrial motility was determined in ImageJ according to [73]. Peroxisomes were counted automatically with ImageJ particle analyzer. The percentage of ER crossing mitochondria was assessed after thresholding and binarization of the ER and mitochondrial signal according to [67]. Similarly, mitochondrial Drp1 was obtained by combining total Drp1 signal with a mitochondrial mask created after thresholding and binarization of the mitochondrial signal. Colocalization of P-MLC was assessed similarly

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

Statistical analysis

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

***C. elegans* work**

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

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Author contributions

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

Conflict of Interest

The authors declare no conflict of interest.

Figure legends

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

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

compared to mock treated cells. White arrowheads point at fission examples, yellow arrowheads to looping. Scale bar: 10 μm, insets are enlarged twofold. (E) Quantification of mitochondrial elongation (long /short axis) in mock treated and FCCP treated U2OS cells showing increased elongation in Sept2 depleted cells compared to mock treated cells, mean of four independent experiments ± SEM.

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

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

Sept2 upon Mdivi-1 treatment. (H) Snapshots of a live cell imaging experiment in U2OS cells showing Sept2 (green, Sept2-YFP), Drp1 (red, Drp1-mCherry) and mitochondria (blue, mitotracker DeepRed) during mitochondrial fission. Merged and single channels show the dynamics of Sept2 and Drp1 during mitochondrial fission (yellow arrowheads). Scale bar: 2 μm.

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

Figure 6: Sept2 does not affect mitochondrial Myo2 localization and does not require actin dynamics for interacting with Drp1. (A) Immunofluorescence analysis of P-MLC (green) recruitment to mitochondria (red, Tom20) in mock treated and Sept2 depleted HeLa cells. Insets show twofold enlargements. Scale bar: 10 μm. (B) Colocalization of P-MLC with mitochondria was assessed with Icy software and showed no difference between mock and Sept2 depleted cells. (C) Mock treated or Sept2 depleted Drp1 -/- MEF cells were labeled with mitotracker orange (red), treated for the indicated amount of time with 2μM FCCP and stained for actin with phalloidin (green). Insets are enlarged twofold. (D) Immunofluorescence analysis of the actin binding proteins Arp3 and cofilin (green), colocalized with cytocrome c (red) and cortactin (green), colocalized with Hsp60 (red) in mock treated or Sept2 depleted Drp1 -/- MEF cells. Scale bars: 10 μm (E) Cytosol and crude mitochondria fractions were prepared from mock treated or Sept2 depleted Drp1 -/- MEF cells and analyzed by western blotting with the indicated antibodies. (F) HeLa cells were mock treated or treated with cytochalasin D and subjected to Sept2 immunoprecipitation. Immunoprecipitates were

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

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

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

Expanded View Figures

Expanded View Figure legends

Figure EV1 : Western blot quantification of septin depletion. Sept2 depletion affects mitochondrial morphology but not ER-dependent mitochondrial fission. (A) Western blot showing siRNA mediated silencing of septins. Sept2 is most efficiently depleted upon Sept2 siRNA. Sept7 siRNA also codepletes Sept2, while Sept9 siRNA does not affect Sept2 levels. (B-D) Quantification of Sept2, Sept7 and Sept9 protein level after siRNAs treatment, mean of at least three independent experiments. The red line serves as a reference, and highlights the minimal residual amount of Sept2, obtained upon treatment with Sept2 siRNA. (E) Representative images of Sept2 depleted HeLa cells showing mitochondrial elongation induced by two different siRNAs. (F) Quantification of the percentage of cells showing mitochondrial hyperfusion after treatment with each Sept2-targeting siRNA, n>150 cells from two independent experiments. (G) Quantification of ER-marked mitochondrial fissions in control and Sept2 siRNA treated cells according to [67].

Figure EV2 : Analysis of mitochondrial motility and fission/fusion protein levels upon Sept2 depletion (A) Total lysates of mock treated or Sept2 depleted cells were analyzed by western blot and probed for Mfn1, Mfn2 and Drp1. (B) Western blot quantification of total Mfn1, Mfn2 or Drp1 levels in mock treated and Sept2 depleted total cell lysates, mean of two independent experiments. (C) Mock treated cells and Sept2 depleted cells were labeled with mitotracker orange and imaged on a spinning disk microscope for 10 minutes. Ten snapshot images, taken every 15s, were overlaid and colour-coded to visualize mitochondrial motility, with immotile mitochondria appearing white. (D) Quantification of mitochondrial motility according to [73] ; ten individual timepoints in two independent experiments expressed as a mean ±SEM. Scale bar: 10 μm.

Figure EV3 : Sept2 localises to mitochondria and interacts with Drp1 in different cell types (A) Total HeLa cell lysate as well as crude mitochondrial fraction and postnuclear supernatant were first analysed for mitochondrial enrichment by western blotting with Drp1, tubulin and Tom20 antibodies and then equal amounts were

reloaded and probed for Sept2. (B) Confocal (left panel) and Elyra SIM (right panel) image of U2OS cells labelled with Tom20 (red) and Sept2 (green). Insets show fourfold enlargements. Arrowheads show small Sept2 fibers colocalizing with mitochondria. Scale bar: 10 μm. (C-D) Co-immunoprecipitation of endogenous Drp1 with endogenous Sept2 in HEK293 (C) and U2OS (D) cells. (E) Immunoprecipitation of endogenous PI4KIIα in HeLa cells shows no co-immunoprecipitation of Drp1. (F) Immunofluorescence analysis of HeLa cells treated for 1 h with Mdivi-1 and stained with Tom20 to reveal mitochondrial morphology. Mdivi-1 treatment increased mitochondrial interconnectivity as previously described [51]. (G) GST pulldown of recombinant Sept2 with GST-Drp1 or GST alone in the presence or absence of 50μM Mdivi-1, which partially decreased Sept2-Drp1 interaction.

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

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

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

Expanded view tables

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

Table EV2 : Plasmids

Plasmid	Oligos	reference
Sept2 HA – rescue	cgtttatgaa**C**GC**G**AT**T**CACAACAAGGTGaatattgtg (siRNA targeting sequence bold uppercase, silent mutations in red)	This study
Sept2 HA – dGTP	aaaaattgaaaga**GGT**gtccagattgaggc (T78G mutation bold uppercase)	This study
Sept7-HA		Ribet et al, in preparation
Sept2-YFP		[68]
OM-GFP		[48]
Drp1-mCherry		[67]

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]
PI4KIIα	in house production	[80]

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