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► **To cite this version:**

Koyomi Nakazawa, Briec Chauvin, Stéphanie Mangenot, Aurélie Bertin. Reconstituted in vitro systems to reveal the roles and functions of septins. *Journal of Cell Science*, 2023, 136 (19), pp.jcs259448. 10.1242/jcs.259448 . hal-04236730

HAL Id: hal-04236730

<https://cnrs.hal.science/hal-04236730>

Submitted on 11 Oct 2023

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Reconstituted *in vitro* systems to reveal the role and functions of septins

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Abstract

Septins are essential cytoskeletal proteins involved in key cellular processes and have also been implicated in diseases from cancers to neurodegenerative pathologies. However, they have not been as thoroughly studied as other cytoskeletal proteins. *In vivo*, septins interact with other cytoskeletal proteins and with the inner plasma membrane. Hence, bottom-up *in vitro* cell-free assays are well suited to dissect the roles and behavior of septins in a controlled environment. Specifically, *in vitro* studies have been invaluable to describe the self-assembly of septins into a large diversity of ultra-structures. Given that septins interact specifically with membrane, the details of these septin membrane interactions have been analyzed using reconstituted lipid systems. [In particular, at a membrane, septins](#) are often localized at curvatures of [micrometer](#) scale. In that context, *in vitro* assays have been performed with substrates of varying curvatures (spheres, cylinders, undulated substrates) to probe the curvature sensitivity of septins. [This review](#) will first present the structural properties of septins in solution and discuss the interplay of septins with cytoskeletal partners. We will then discuss how septins interact with biomimetic membranes and induce their reshaping. We will finally highlight the curvature-sensitivity of septins and how they alter the mechanical properties of membranes.

Introduction

To reveal specific molecular mechanisms within cells, bottom-up reconstituted systems can be particularly useful. Indeed, the interpretation of effects of overexpression or down-regulation of [specific](#) genes in the cell can be complicated because of the numerous genes expressed in human cells (about 19,000) and the complex regulation of their expression (Ezkurdia et al., 2014). In addition, the high protein concentration within the cytosol [200-300 mg.mL⁻¹, (Milo, 2013)] further complicates the dissection of underlying molecular mechanisms. In this context, it is crucial to be able to analyze the role of a specific protein and its partners. Reconstituted *in vitro* systems, in which the environment and the parameters can be controlled to a large extent, are thus often an approach of choice, and in fact can be essential to reveal the underlying molecular functions.

Septins are cytoskeletal proteins; they are ubiquitous in most eukaryotes and essential to many cellular processes. Septins are required for cytokinesis and the completion of cell division (Addi et al., 2018; Finger, 2005; [Hartwell, 1971](#); Spiliotis et al., 2005). In budding yeast, [septins are found at the division neck of dividing cell \(Fig. 1, left\)](#), and mutations or deletions of septins lead to the formation of multi-nucleated buds and impaired cytokinesis (Haarer and Pringle, 1987; Kim et al., 1991; Versele et al., 2004). Within higher eukaryotes, septins are [also](#) found at the intercellular bridge in between dividing cells. They form ring-like structures on both sides of the midbody and are essential for the final membrane scission (Karasmanis et al., 2019; Renshaw et al., 2014; Spiliotis et al., 2005; [Surka et al., 2002](#); Wang et al., 2019). [In addition, in higher eukaryotes, septins appear to have a key role in various tissues. For instance,](#) septins are required for neuro-morphogenesis ([Fig. 1, middle](#)) (Cho et al., 2011; Marttinen et al., 2015a; [Tada et al., 2007](#); [Xie et al., 2007](#)) and spermiogenesis ([Fig. 1, right](#)) (Lin et al., 2011). Because they exert multiple roles, septins have also been implicated in the development of common diseases (Hall and Russell, 2004). For instance, septin overexpression or downregulation occurs in cancerous tumors (Angelis and Spiliotis, 2016; James et al., 2019; Montagna et al., 2003), and SEPT9 is indeed used as a biomarker in colon cancer ([Kamel et al., 2022](#)). Septin misregulation has also been linked to neurodegenerative diseases (Marttinen et al., 2015b). Septins have not been as thoroughly studied as other cytoskeletal proteins, but because of their emerging importance in essential cellular processes, they have gained increased attention more recently.

Septins are known to interact with different partners, as well as their cytoskeletal counterparts actin and microtubules ([Kinoshita et al., 1997](#); [Surka et al., 2002](#)). However, their role when bound to microtubules and actin is still unclear (Spiliotis, 2018). [Septins are membrane-binding proteins and were shown to localize to the membrane by using immune-gold labelling methods \(Ewers et al.,](#)

2014; Kinoshita et al., 1997). At membranes, septins are thought to form a scaffold and recruit essential partners (Chant, 1996; Kinoshita, 2006). In addition, they are involved in assembling diffusion barriers for membrane-bound components (Takizawa et al., 2000). Indeed, a recent report compared different potential diffusion barriers of fluorescently tagged membrane-bound components, and from their analysis, the authors conclude that septins display the strongest compartmentalization effect (Pacheco et al., 2023). It is likely that steric hindrance caused by the filaments restricts the diffusion of large membrane-bound components. This, however, remains to be fully tested. Interestingly, at the membrane, septins are often localized at micrometer-scale negative gaussian curvatures (see text Box 1), such as those at the bases of cilia or dendrites (Beber et al., 2019b; Bridges et al., 2016; Cannon et al., 2017; Cannon et al., 2019; Kinoshita et al., 2002), suggesting that septins might be able to sense micrometer-scale curvatures; this is in contrast to other curvature-sensing proteins such as BAR proteins that usually sense nanometer-scale curvatures (Simunovic et al., 2019).

Septins form complexes that are composed of several subunits, with each being present in two copies with a palindromic organization (Barral and Kinoshita, 2008; Marquardt et al., 2019). The number of possible septin subunits depends on the organism, ranging from two in *Caenorhabditis elegans*, five mitotic species in *Saccharomyces cerevisiae* to thirteen subunits in *Homo sapiens* (Fig. 2A). In humans, the 13 different septins (SEPT1 to -12 and SEPT14) are differentially expressed in specific tissues. They are found as either hexamers or octamers (Fig. 2B), whereas in budding yeast, septin complexes are octameric (Lindsey and Momany, 2006) (Fig. 2B). The groups proposed by Momany (Shuman and Momany, 2022) and Kinoshita (Kinoshita, 2003) classified septins according to their sequence similarities (Fig. 2A). Kinoshita group first classified septins from yeast to animal. The Momany lab later classified fungi septins. Subunits within an oligomer can be exchanged with subunits belonging to the same group. Hence, multiple combinations of septin subunits are possible to give rise to various complexes, and with potentially different functions and association with specific diseases. For example, Sept4 is involved in sterility (Ihara et al., 2005) and Sept8 in Myelin outfoldings in the nervous system (Patzig et al., 2016) (for reviews see: (Dolat et al., 2014; Mostowy and Cossart, 2012)). In addition, some of the septins such as human Sept9 can be expressed as different splice variants which can exhibit different functions (Connolly et al., 2011; Devlin et al., 2021; Estey et al., 2010; Verdier-Pinard et al., 2017)

Given their multi-tasking nature and implication in diseases, it is important to characterize and reveal the functions of specific septin complexes. To dissect the specific role and behavior of distinct septin complexes, defined bottom-up in vitro assays are highly valuable. The interaction between septins and partners can for instance be probed in vitro by pelleting assays (Bai et al., 2013). Until recently,

the co-expression of septins remained challenging especially for human complexes. Using bottom-up assays and devices of different curvatures (spheres, cylinders, undulated substrates), a specific and peculiar septin curvature sensitivity has been described (Beber et al., 2019b; Bridges et al., 2016; Cannon et al., 2019; Nakazawa et al., 2023). The molecular and/or biophysical mechanisms underlying this sensitivity remain to be clarified. Because septins have flexible domains, elucidating their detailed structure, and within complexes remain challenging. Indeed, septin complexes do not crystallize easily and, up-to-date, only a single human septin complex has been crystallized that provided sufficient resolution (Sirajuddin et al., 2007), and the use of cryo-EM could thus be promising (Mendonça et al., 2021). The functions and mechanistic regulation of septins have not been as thoroughly investigated as that of other cytoskeletal elements, such as actin or microtubules. Nevertheless, some efforts have been directed towards the elucidation of septin functions using *in vitro* reconstituted assays. Furthermore, because *in vivo*, septins interact with multiple partners, it is also important to analyze these interactions in detail *in vitro*.

To this end, functional septin complexes must be first purified in a reproducible manner, so that their ultrastructural features can be investigated. This Review will therefore first describe the protein expression/purification methodologies and the resulting structural data obtained by X-ray crystallography and electron microscopy, and describe the species-specific ultrastructural organization of septins. We will then discuss how the septin-cytoskeleton and septin-membrane interaction can be assayed to investigate the dynamics and organization of septins when bound to partners. In particular, we highlight the curvature sensitivity of septins and the possible resulting membrane deformations will be presented.

Analysis of septin self-assembly *in vitro*

Structure: From single subunit to oligomeric complex

Before septins could be recombinantly expressed and purified, immunoaffinity strategies were used to extract septin complexes from budding yeast (Frazier et al., 1998) and *Drosophila* (Field et al., 1996); this allowed to elucidate the nature of the septin subunits involved in the complex self-assembly as well as the filamentous nature of septins *in vivo*. In 2002, human septin complexes were extracted from brain tissues and cultured cells, and expressed, for the first time as a hexameric human septin complex in insect cells (Kinoshita et al., 2002). Septin complexes from any organism were first successfully expressed and purified by inducing the production of all proteins simultaneously to facilitate proper refolding and complex assembly instead of expressing the

proteins individually and attempting to reconstitute the complexes a posteriori (Bertin et al., 2008; Farkasovsky et al., 2005; Kinoshita et al., 2002; Sheffield et al., 2003; Sirajuddin et al., 2007). However, the design of constructs and choice of location of affinity tags was not straightforward. To generate octameric budding yeast septins, the four septin genes that are present in two copies were expressed within two expression vectors encoding genes of each of the two septin subunits, before being subjected to established purification (Bertin et al., 2008; Garcia et al., 2011). For human septins, single subunits or hexamers were first expressed and purified (Kinoshita et al., 2002; Mavrakis et al., 2014; Sirajuddin et al., 2007). However, *in vivo*, human septins appear to be functional as octameric complexes (Martins et al., 2023). Human septin octamers have thus been generated, in turn, displaying different splice variants for Sept9 (lv et al. 2021). Most of the designed plasmids are available through the “addgene” website (<https://www.addgene.org>).

Structural features of septins have thereby been deciphered. The first high resolution crystal structure was that of a hexameric human septins complex comprising SEPT2-SEPT6-SEPT7 at about 4 Å resolution (Sirajuddin et al., 2007). Following this seminal report, structures at higher resolutions (2 to 3 Å) of individual septins were described, including of several human septin subunits, such as SEPT7 (Serrão et al., 2011; Zent et al., 2011) and SEPT3 (Macedo et al., 2013), as well as of budding yeast septin Cdc11 (Brausemann et al., 2016) or *Chlamydomonas* CrSEPT (Pinto et al., 2017). As expected from their sequence, septins display a central RAS-like structure flanked by flexible, unstructured domains (Sirajuddin et al., 2007) (see Fig. 2C). The central RAS and GTP-binding (G) domain is the most conserved. It comprises six beta sheets surrounded by alpha helices. The G1 sub-domain contains a P-loop, while G3 is made of a switch-2 structural domain. The G4 domain selectively binds guanine nucleotides. The G domain encompasses the so-called septin unique element (SUE) of 50 residues that is unique for a specific septin. The N-terminal domain of the septins is more variable and disordered. At their C-terminus, septins display typical heptad repeats capable of undergoing coiled-coil assemblies. All purified septin complexes so far, including from *C. elegans* (John et al., 2007), budding yeast (Bertin et al., 2008), *Ashbya gossypii* (Kaplan et al., 2016), *Drosophila* (Field et al., 1996; Mavrakis et al., 2014) and human (Sirajuddin et al., 2007), show similar and conserved features. They all self-assemble into rod-like complexes that include two copies of each septin subunit under non-polymerizing conditions (*i.e.* ionic strengths higher than 150 mM and in the absence of membrane) (Fig. 2B). Regardless of the actual septin complex, the septin rod always displays a palindromic arrangement of the subunits with an axis of symmetry in the center. For instance, budding yeast septins organize as Cdc11-Cdc12-Cdc3-Cdc10-Cdc10-Cdc3-Cdc12-Cdc11 with Shs1 being a possible alternative to Cdc11 (Fig. 2A and B), and an example of a human septin octamer is Sept2-Sept6-Sept7-Sept9-Sept9-Sept7-Sept6-Sept2 (Fig. 2B). These arrangements were

determined by using specific tags for given subunits whose position was analyzed based on electron microscopy imaging (Bertin et al., 2008; John et al., 2007; Mendonça et al., 2019). [The arrangement of the human septin hexamer and octamer had, unfortunately, been determined incorrectly initially and was only revised about ten years later \(McMurray and Thorner, 2019\).](#) In addition, the interfaces between each septin subunit alternate between so-called G interfaces and N-C interfaces (Fig. 2B). G interfaces are mediated through GTP-binding domains, while N-C interfaces are mediated by alpha helices from the N- and C-terminus of a septin subunit. N-C interfaces mediate the central interaction, as well as the terminal interaction, thus enabling septin polymerization ([see Text Box 2](#)). Polymerization occurs through an end-to-end interaction of octameric rods in conditions where the ionic strength is decreased. In addition, filamentous elongation can be coupled with filament lateral pairing through coiled coil interaction (Bertin et al., 2008).

However, in the crystal structures, only the structured domains are well resolved, while the terminal domains that are unstructured are not. The flexible regions at the C-terminus, ([see Fig. 2C](#)) have been predicted to be able to arrange into coiled-coil domains (Sala et al., 2016). Recently, some of the coiled coil domains were crystallized individually (Leonardo et al., 2021). Within a typical heptad coiled-coil sequence, the first and fourth residues usually are hydrophobic (Lupas and Gruber, 2005). However, in the case of septins, the first amino acid of the heptad repeat is hydrophilic and not hydrophobic, and this peculiar configuration can give rise to either a parallel or antiparallel arrangement of the coiled coils in the homodimer. Hence, coiled coils can result in either long range (25 nm) or short range (5 nm) filament pairing. Moreover, in an anti-parallel coiled-coil arrangement, a hydrophilic surface would be exposed that could possibly interact with the membrane (Leonardo et al., 2021).

Although a number of crystal structures of individual septin subunits have been obtained, only one septin oligomer (Sept2-Sept6-Sept7) has been crystallized (Sirajuddin et al., 2007). Recently, the hexameric septin complex has been described at 3.6 Å resolution under more physiological conditions by using cryo-EM (Mendonça et al., 2021). Cryo-EM imposes fewer mechanical constraints on a protein and more accurately reflects the protein environment in a hydrated state and the global conformation of a protomer, following its vitrification in ice, thus typically differs from the crystal structure. Interestingly, in the cryo-EM structure, the hexameric septin rod displays a peculiar kink within its center, at the interface between Sept7 and Sept7. This kink results in a 22 Å lateral deflection of the terminal subunit from the center of the rod (Mendonça et al., 2021). This bent infers some flexibility and could reflect an intrinsic curvature of the septin filament.

Ultrastructure: From oligomers to filaments, bundles and gauzes

In vivo, as shown by polarization fluorescence microscopy (Vrabloiu and Mitchison, 2006), septins are able to undergo structural rearrangements at the budding yeast constriction site. Here, septin filaments arrange parallel to the mother bud axis and rotate perpendicularly at the onset of cytokinesis (Vrabloiu and Mitchison, 2006).

Hence the variability of septin self-assembly observed *in vitro* most likely reflects their plasticity in a cellular context. Minimal octameric septin rods tend to self-assemble into filamentous structures in solution under low ionic strength (Bertin et al., 2008), for example, the budding yeast octameric complex containing Cdc11 (Fig. 2D). The kinetics of septin polymerization is fast and completed within seconds (Bertin et al., 2010). Depending on the actual septin subunit composition, the ultrastructure of the resulting assemblies varies in solution. For instance, budding yeast septins containing Cdc11 instead of terminal Shs1 self-assemble into paired filaments (Fig. 2D, right panel) that resemble railroad tracks below a salt concentration of approximately 150 mM via dimerization of their C-terminal coiled-coil domains (Booth and Thorner, 2016; Booth et al., 2015). At high protein densities, these paired filaments can aggregate into bundles with septin subunits remaining in register (Fig. 2D, middle left panel). Interestingly, replacing Cdc11 with Shs1 in budding yeast results in a dramatic rearrangement of the resulting septin filamentous ultrastructure from straight filaments and/or bundles to bundled rings with an estimated diameter of approximately 1 μm (Fig. 2D, middle right panel) and also to a gauze-like mesh (Fig. 2D, right panel) (Garcia et al., 2011; Taveneau et al., 2020). Septins from higher eukaryotes, such as *Drosophila* (Field et al., 1996; Mavrakis et al., 2014) or humans (Iv et al., 2021; Kinoshita, 2003; Kinoshita et al., 2002; Martins et al., 2023) bundle spontaneously, without any paired filaments identifiable in solution. Human septin complexes were also shown to self-assemble into ring-like bundles (Iv et al., 2021; Kinoshita et al., 2002), similarly to the Shs1-containing budding yeast septin complexes. Observations from independent experiments indicate that septins are able to arrange into different higher-order structures, which might reflect their varying cellular roles. Although most of the analyses performed using imaging or bulk measurements (Bertin et al., 2008; Booth and Thorner, 2016) have shown that septins polymerize at low ionic strengths, a recent study carried out by AFM shows that filaments could be generated in the presence of higher salt concentrations on mica substrates at low pH (Jiao et al., 2020). This study aimed to monitor septin polymerization in real time by using high speed AFM. This discrepancy to previous findings might result from the substrate used (mica). In these conditions, the c-terminal extension of Cdc11 in yeast septin octamer contributes to filaments pairing more than those from Cdc12 and Cdc3. This suggests that a charged environment might alter septin self-assembly. Several reports studied the polymerization of septins in real time assayed using

supported lipid bilayers deposited on a silica (Szuba et al., 2021), mica (Jiao et al., 2020) or glass substrates (Bridges et al., 2014).

To conclude, septin complexes of different species share common and conserved features at the molecular level. However, sequence variation between species results in variety of architectures upon septin self-assembly and polymerization, which might reflect their various functions and promote their interaction with numerous partners.

Interaction of septins with cytoskeletal partners

As mentioned above, *in vivo*, septins associate with both actin filaments and microtubules (Kinoshita et al., 1997; Kinoshita et al., 2002; Nagata et al., 2003; Spiliotis and Nakos, 2021; Surka et al., 2002), as well as with anillin (Kinoshita et al., 2002). Specifically, in cells depleted of filamentous actin (treated with cytochalasin), septins self-assembled into ring-like structures (Kinoshita et al., 2002). To dissect the interplay between septins and other cytoskeletal filaments, *in vitro* studies mixed purified septins with either actin or tubulin, for instance, reconstituted actin filaments were mixed with either fly septins (Pnut-Dsep1-Dsep2)(Mavrakis et al., 2014) or human septin hexamers (SEPT2-SEPT6-SEPT7) (Kinoshita et al., 2002; Mavrakis et al., 2014), showing that septins crosslinked actin filaments into bundles that could be either linear or curved. Indeed, several studies using cultured human cells found that septins colocalize with actin stress fibers (Dolat et al., 2014; Kinoshita et al., 2002; Martins et al., 2023; Salameh et al., 2021; Surka et al., 2002). Independent *in vitro* studies have also analyzed the interaction of septin SEPT9 with microtubules. One study showed that upon mixing of microtubules with SEPT9, microtubules bundle and grow longer (Bai et al., 2013). To better understand this elongation, subsequent work studied the dynamics of microtubule elongation in the presence of SEPT9 at different concentrations (Nakos et al., 2019). These authors found that at concentrations of a few hundred nanomoles, SEPT9 strongly decreased the rate of microtubules catastrophes, thereby giving rise to elongated microtubules (Nakos et al., 2019). More recently, isoform 1 of Sept9 and its MAP-like motif have been shown to interact specifically with microtubules both *in vivo* and *in vitro* (Kuzmić et al., 2021). Sept7 was also shown to interact with microtubules *in vitro* and in neuronal tissue (Hu et al., 2012). *In vivo*, BORG proteins, Cdc42 effectors, control the localization of septins to either actin stress fibers or microtubules (Calvo et al., 2015; Salameh et al., 2021). However, thus far BORG proteins have not been added to *in vitro* assays together with septins.

The interplay between septins and cytoskeletal partners has only been begun to be considered and further work both *in vivo* and *in vitro* is needed to understand their respective roles and to uncover possible novel septin functions.

Septin interactions with the membrane

Septin on biomimetic membranes

In vivo, using immuno-gold labelling in neurons, septins were shown to localize in the vicinity of membranes (Ewers et al., 2014). Super-resolution microscopy performed in fungi also highlighted the presence of septin filaments close to membranes (Kaplan et al., 2015). In addition, human septins are in close vicinity to the plasma membrane when they are bound to actin stress fibers and thus have been proposed to anchor actin to the membrane (Martins et al., 2023). At the membrane, septins assemble into a scaffold (Kinoshita, 2006), where they can form diffusion barriers (Caudron and Barral, 2009; Ewers et al., 2014; Hu et al., 2010; Schmidt and Nichols, 2004) and also have a role in the generation of blebs and protrusions (Gilden and Krummel, 2010; Gilden et al., 2012). Therefore, it is essential to study the organization and behavior of septins on lipid membranes. So far, the architecture of septin filaments on membranes *in vivo* has barely been addressed. However, it appears that septins on membranes have a tendency to arrange into meshed networks, or gauzes, of orthogonal filaments (Fig. 3A) as determined by electron tomography (Bertin et al., 2012) or SEM of spheroplasts (Ong et al., 2016), or into parallel sets of filaments in *A. gossypii* (Bridges et al., 2016).

A number of bottom-up investigations have focused on trying to understand how septins interact with membranes (Bertin et al., 2010; Bridges et al., 2014; Bridges et al., 2016; Szuba et al., 2021; Tanaka-Takiguchi et al., 2009; Taveneau et al., 2020) or how they polymerize when bound to a reconstituted membrane (Bertin et al., 2010; Bridges et al., 2014; Szuba et al., 2021). Because septins often specifically localize at micrometre-scale membrane curvatures, how septins can reshape or deform membranes has also been investigated (Beber et al., 2019b; Tanaka-Takiguchi et al., 2009). By using purified lipids tagged with fluorescent probes, it is possible to reconstitute artificial membranes to mimic the cellular environment with a defined lipid composition. These generated membranes can be either flat, supported non-deformable membranes, either monolayers or bilayers, or deformable vesicles of different sizes, such as giant unilamellar vesicles (GUVs; several micrometers to tens of micrometers in diameter) or large unilamellar vesicles (LUVs; several hundreds of nanometers in diameter). It has been shown that the interaction of septins with membrane is favored when phosphoinositides and particularly PI(4,5)P₂ are present in biomimetic

membranes (Beber et al., 2019a; Bridges et al., 2014; Zhang et al., 1999). Specifically, by using mitotic budding yeast septins, in the absence of PI(4,5)P₂, the recruitment of septins to GUVs is impaired, even when other negatively charged lipids (1,2-dioleoyl-sn-glycero-3-phospho-L-serine, DOPS) are present (Beber et al., 2019a).

Based on several independent studies, it appears that the septin arrangement on biomimetic membranes reflects, at least partially, that in the cell. For example, budding yeast septins assemble into paired filaments both in spheroplasts (Ong et al., 2016) (Fig. 3A) and in reconstituted *in vitro* model systems (Fig. 3B left). Septin filament assembly is enhanced on lipid monolayers, as visualized by electron microscopy, under conditions that prevent filament elongation in solution (Bertin et al., 2010). Human and/or *Drosophila* septin protomers tend to self-assemble into massive bundles in solution (Kinoshita et al., 2002; Mavrakakis et al., 2014; Szuba et al., 2021), suggesting that lateral interactions between filaments is prevalent. Septin filaments bound to membranes tend to organize within sheets of parallel filaments arranged as a liquid crystalline-like nematic sheet (Fig. 2B, right) (Beber et al., 2019b; Bertin et al., 2010) or meshworks of orthogonal networks of filaments (Fig. 3B, middle) (Beber et al., 2019b; Bertin et al., 2010; Garcia et al., 2011; Nakazawa et al., 2023; Ong et al., 2016). In the presence of membranes, only a maximum of two layers of filaments were observed by either AFM or electron microscopy (Fig. 3B right) (Szuba et al., 2021). The authors suggest that the coiled-coil domains are crucial in promoting the two-layered septin self-assembly. Hence, the interaction of septins with lipids compete and prevail over lateral septin-septin interactions observed upon filament bundle formation in solution (Szuba et al., 2021). To obtain further insights into septin-membrane interactions, higher resolutions methods, using for example cryo-EM methods coupled to cutting edge image processing will need to be used.

Another important aspect is the dynamics of septins on membranes, which has been barely investigated. By using fluorescently labelled septins, one study has shown that septin filaments polymerize on lipid membranes bilayers by annealing (Bridges et al., 2014). Here, by using live fluorescence microscopy, end-to-end filaments interaction were shown to result in their fusion. Moreover, filaments were also observed to fragment, suggesting that an equilibrium length can be reached, depending on the septin density (Bridges et al., 2014). A recent study has shown that septins, already bound to membranes, further facilitate further interaction of additional bulk septins to the membrane, meaning that there is cooperative binding (Shi et al., 2023).

Analysis of the curvature sensitivity of septins on membranes

In contrast to most nanometer-scale curvature-sensitive proteins (BAR proteins, for instance), septins appear to sense lower membrane curvatures in the micrometer range. Two models for the

underlying molecular basis have emerged: a molecular model implicating amphipathic helices and a geometric model owing to the filamentous properties of septins. However, [these two models are not contradictory, and most likely both contribute to the curvature-sensing behavior](#). The first molecular model, based on work by the group of Amy Gladfelter, emphasizes the contribution of an amphipathic helix in micrometer-scale curvature recognition (Cannon et al., 2019). They used silica beads of different diameters and thus curvatures decorated with lipid bilayers to determine septin fluorescence intensities as a function of bead diameter and showed that a positive curvature favors interactions between septin and the bead, with a preferential curvature sensitivity of $2 \mu\text{m}^{-1}$ (Bridges et al., 2016). Furthermore, by using cylinders of various diameters onto which a lipid bilayer has been deposited, a preferential orientation of septin filaments when bound to cylinders was observed, either aligned with the axis of the cylinders or perpendicular to it (Cannon et al., 2019) ([Fig. 3C, left and middle panel](#)). Here, the mean orientation corresponds to an apparent radius of curvature of the septin filament that is higher than the radius of the rod. For cylinders with a radius of 200 nm, septins orient along the main axis of the cylinder, while increasing the radius induces a tilting of septin filaments until they extend perpendicular to the cylinder axis for radii above 1 μm . Based on septin sequence and use of mutant variants, including truncation of the C-terminal domain of Cdc12, the authors proposed that an amphipathic helix at the septin C-terminus is responsible for this curvature sensitivity (Cannon et al., 2019) [in addition to be most likely required to induce interaction with the membrane \(Fig. 3D\)](#). Another *in vitro* study using *Shigella flexneri* mixed with recombinant septin hexamer shows that septins build a cage around *S. flexneri* cells, and suggests that amphipathic helix domain (of SEPT6) is important for the curvature sensing (Lobato-Márquez et al., 2021). However, the mechanism relying on amphipathic helix domain does not explain the sensitivity of septins to flatter curvatures, as amphipathic helices typically sense nanometer-scale curvatures as in the case for BAR proteins. Another study from the same group suggests that the Shs1 containing budding yeast septin complex is also curvature sensitive owing to an amphipathic helix (Woods et al., 2021).

Together with collaborators, our group proposed an alternative geometric model that supports the intrinsic biophysical properties of septin filaments as the basis underlying this particular curvature sensitivity. To assess the effect of both positive and negative curvatures, we generated wavy undulated substrates with one axis exhibiting alternatively concave and convex geometries, and the other axis without any curvature ([Fig. 3C, right panel](#)) (Beber et al., 2019b). After deposition of a lipid bilayer, the interaction of septins with the substrate was imaged with scanning electron microscopy (SEM) and exhibited a clearly oriented pattern. Septin filaments bound to concave curvatures (negative) oriented in the direction of the curvature, whereas on positive curvatures, they oriented in the direction of zero curvature to avoid bending. This suggests that septins sense or interact more

favorably with negative curvatures, avoiding positive curvatures. This behavior was modeled using a simple geometrical assumption based on the energy of the septin-membrane interaction and septin filament flexibility, and shown to only rely on the filamentous nature of septins (Fig. 3E) (Beber et al., 2019b). Indeed, when using a mutant lacking the N-terminus of Cdc11, and thus unable to polymerize, no specific orientation on those wrinkled substrates could be observed.

Based on this, we can conclude that curvature sensitivity might result from several factors, both at the molecular level, *i.e.* mediated through the amphipathic helix, and at the filament level, based on the intrinsic biophysical properties of septin filaments. **Two distinct factors can thus induce curvature sensitivity synergistically. However, it is still not clear which one is predominant. For instance, preventing polymerization might cause the decrease of interaction between septins and membranes (Bridges et al., 2016). Similarly, mutations of the amphipathic helices may also hinder, to some extent, polymerization, when mutated and thus prevent their assembly and localization at hyphal branches in *A. Gossipy* (Cannon et al., 2019). In addition, sensing positive and negative curvatures might also derived from distinct mechanisms. Lastly, recent reports demonstrated different septin association rates on membrane depending on microscale membrane curvatures. This highlights the importance of the septin-binding kinetics to their curvature-sensing capacity (Cannon et al., 2019; Shi et al., 2023).**

It will be interesting to study septin organization at curvature in both axes (**non-null gaussian curvatures**), which would more closely mimic the geometries encountered in cells, as this might provide further insights into the role of membrane curvature in septin organization.

Septins can reshape membranes

Other cytoskeletal filaments are capable of deforming membranes in the presence of motor proteins (Keber et al., 2014; Tsai and Koenderink, 2015). For instance, actin is involved in the formation of membrane protrusions (lamellipodia and filopodia) during cell migration (Innocenti, 2018).

In the cell, septins are often associated with membrane-remodeling events. For instance, during cell division, micropinocytosis and endocytosis, septins are present when the plasma membrane undergoes either constriction, scission, or budding (Bezanilla et al., 2015). Here, it appears that septins can exert a stabilizing effect on membranes, as septin-depleted cells undergo spontaneous tubulation and display an abnormally high density of protrusions (Gilden et al., 2012). Septins are also involved in stabilizing the myelin membrane in neurons, as their depletion leads to myelin unfolding (Patzig et al., 2016).

Several studies have thus examined whether septins can also reshape membranes *in vitro*, and deformable liposomes have been used to assess the role of septins in membrane deformations in a controlled environment. In a seminal experiment, using cell extracts containing multiples proteins, human septins were shown to induce outward tubulations from GUVs that are fully covered with septin filaments (Fig. 4, left panel) (Tanaka-Takiguchi et al., 2009). When purified human septins are used, a lower density of tubules was also generated, suggesting that septins alone are sufficient for GUV deformation, even though additional factors present in cell extracts might enhance this (Tanaka-Takiguchi et al., 2009). Subsequent work addressed whether this effect is conserved, and indeed, mitotic budding yeast septins also reshape membranes (Fig. 4, middle and right panel) (Beber et al., 2019b). When bound to the outside of GUVs, yeast septins induce inward deformations that are periodically arranged and display spikes every four to six micrometers. In contrast, smaller liposomes with a diameter below 1 μm (LUVs) undergo a significant flattening upon interaction with septin filaments. Cryo-electron tomography shows that these septin-bound liposomes eventually fully flatten to a thickness of approximately 50 nm, with septin filaments extending flat over the membrane (Beber et al., 2019b). The micrometric curvature sensitivity of septins, mentioned above, could explain these membrane deformations of LUVs, as in the presence of a highly positively curved membrane, septins are expected to flatten the liposome in order to remain in a straight configuration. In principle, septin filaments could induce negative curvatures (*i. e.* an inward bending of the membrane), but the dimensions and thus curvatures of LUVs are too small and too curved to allow for the type of deformations that are induced on GUVs. In contrast, when bound to GUVs, septins can deform membranes and induce negative curvatures in the micrometer range by adopting a negative curvature. *In vivo*, septins might be recruited preferentially to micrometric curvatures, such as constriction sites, the base of cilia (Hu et al., 2010), base of dendrites (Tada et al., 2007) or spermatozoa annulus (Lin et al., 2011) by specific factors, for instance, Cdc42 at yeast bud necks. At these favorable curvatures, septins might, in turn, either stabilize the membrane morphology and/or be involved in its reshaping, for instance in cytokinesis (Karasmanis et al., 2019). In addition, septins have been shown to significantly disrupt lipid bilayers that are deposited on mica as observed by fluorescence and atomic force microscopy; here septins indeed reshape lipid bilayers and induce the formation of tubules or doughnut-shaped lipidic objects (Vial et al., 2021). Taken together, these observations demonstrate that septin filaments, without any source of energy, can spontaneously reshape and deform membranes.

Conclusions and future perspectives

Studying the biophysical properties of septins *in vitro*, specifically when bound to membranes, has considerably enhanced our understanding on their possible functions and roles *in vivo*. The observed ability of septin filaments to give rise to different ultrastructures likely reflects the multiple functions of septins. Septins are able to deform membranes in a specific manner that directly correlates with their curvature sensitivity, which results from both their specific secondary structure as well as intrinsic biophysical features of the filaments.

As a next step, these *in vitro* findings will need to be correlated to septin-induced phenotypes *in vivo*, and here, additional bottom-up assays are still valuable in assessing additional septin functions as they allow to [decouple the function of septins from that of any other factors](#). However, such [simplified *in vitro* assays cannot fully recapitulate the cellular context and thus need to be performed in parallel to cell biology experiments, or at least using the knowledge obtained from *in vivo* findings](#). For instance, outstanding questions are the mechanical properties of septins (see text box 3), as well as their actual role in the establishment of diffusion barriers. Furthermore, the ultrastructure of septins needs to be determined in a cellular context, for instance using novel high-resolution imaging and labelling methods.

Taken together, even though significant progress has recently been made in studying septins *in vitro* using bottom-up assays, a lot remains to be explored to reveal the cellular role of those ubiquitous cytoskeletal filaments.

Text boxes

Text Box 1 Septin location *in vivo* and definition of curvatures

Curvature can be defined either by Gaussian Curvature K or Mean Curvature H . Gaussian curvature and mean curvature present the product or average of principal curvatures in two orthogonal directions of a two-dimensional surface appearing in three dimensions, respectively (see box figure B). Unlike mean curvature, gaussian curvature describes how much the surfaces are similar to spheres or horse saddle shapes.

Text Box 2 Hierarchical structure of septin self-assembly

Septin self-organization is conserved among species. They assemble into complexes comprising from between two to four distinct septin subunits. The minimal complexes are palindromic rods displaying an axis of symmetry. Hence each of the septins subunits are present in two copies in heteromeric complexes (see box figure). *In vitro*, these complexes disperse into solution at high ionic strength (above 150 mM salt). Under lower salt conditions, however, the minimal rods assemble into a variety of filamentous structures from paired filaments, bundles, rings of bundles or gauzes of orthogonal filaments (see box figure).

Text Box 3 Septin mechanical properties and its relation with their self-assembled structures

The mechanical properties of cytoskeletal filaments have an important role in determining cell shape and rigidity, and the persistence length of a filament usually reflects its rigidity. The flexibility of septin filaments has been estimated from persistence length measurements based on measurements similar to those performed for both actin and microtubules. Depending on the exact study, the persistence length of budding yeast septin filaments was found to vary from 6 to 12 μm (Beber et al., 2019b; Bridges et al., 2016). However, such measurements are performed on either supported lipid bilayers or passivated glass, but to more closely relate to the behavior of septins *in vivo*, it would be more relevant to study the effect of septin filaments on the mechanical properties of membranes as septins interact with the inner plasma membrane. The mechanical properties of membranes are typically determined from estimating their bending rigidities and elastic moduli. Micropipette aspiration and tube-pulling assays of GUVs covered with septins have been performed, but they showed no significant effect of the presence of septins on their bending modulus (Beber et al., 2019b). These unexpected findings clearly indicate that additional experiments need to be performed in order to fully understand the role of septins in membrane mechanics. One can assume that the mechanical properties of septins likely depend on their ultrastructural organization into either gauzes of orthogonal filaments, bundles or sheets of parallel filaments. In addition, these ultrastructures certainly depend on the presence of additional partners. For instance, one of the Cdc42 effector Gic1 interact with one of the yeast septin subunit Cdc10, and can crosslink and stabilize septin filaments (Sadian et al., 2013). It is thus interesting to investigate mechanical properties of septins as a presence of additional interaction partners. Finally, it is important to address how mechanical properties of septin changes depending on the microscale curvatures or geometries that more closely mimic the *in vivo* environment.

Figure legends

Fig. 1 Examples of septin location *in vivo*

Septins localize division neck of dividing cells (left), base of dendritic protrusion and branching points of neuron cells (middle) and annulus of spermatozoid (right).

Fig. 2 Organization of septins from subunits to ultrastructures

(A) Classification of septins across species according to (Shuman and Momany, 2022) and (Kinoshita, 2003). *Homo sapiens* (Hs), *Drosophila melanogaster* (Dm), *Caenorhabditis elegans* (Ce) and *Saccharomyces cerevisiae* (Sc). (B) Schematic illustration of a model heteromeric septin complex. NC-interface and G-domain of each subunit is indicated below in yeast octameric complex, and c-terminal coiled coil is drawn by long-tail with arrows directing the C-end. Septins from the same group can replace each other in the complex. (C) Sequence domains (N-terminal domain, GTP binding domain, Septin unique element, C-terminal Extension holding a coiled coil) of a prototypical septin. (D) (i to iv) Electron microscopy images of self-assembled septin structures in solution. (i) Paired filaments formed from purified Cdc3–Cdc10–Cdc11–Cdc12 hetero-octamers in low salt (50 mM) (ii) These filaments further assemble into bundles as seen in the EM image of a bundled yeast septin complex formed with Cdc11. (iii) Septin filament bundles can also form rings and (iv) gauze-like meshworks with Shs1. Images in (i) and (ii) reproduced from (Bertin et al., 2008) Copyright (2008) National Academy of Sciences. (iii) and (iv) reproduced from (Taveneau et al., 2020).

Fig. 3 Membranes influence the organization of septin filaments. (A) *In vivo*, septin filaments can assemble into a meshwork as shown in the platinum Replica EM PREM image of septin filaments on membrane spheroplasts during cell division. Orthogonal networks are highlighted with two different colors with red arrows pointing at single filaments. Reproduced from (Ong et al., 2014) with permission. The schematic illustration on the right shows the underlying filament composition. (B) EM images of purified Cdc3–Cdc10–Cdc11–Cdc12 hetero-octamers on membranes. Left: In high salt (300 mM), septin assemble into paired filaments on lipid monolayers. Reproduced from (from Bertin et al., 2010). Middle: cryo EM images of septin filament forming an orthogonal network on LUVs; reproduced from (from Beber et al., 2019b). Right: AFM image of human septin filaments, forming a nematic sheet when deposited on lipid bilayers. Reproduced from (Szuba et al., 2021). The right color code shows the scale bar for the height in the image. © 2021 Szuba et al.; published under CC-BY 4.0 license. (C) Septin alignment depends on the curvature of the substrate. Shown here are SEM images of septin filaments deposited on lipid bilayers with different curvatures (left and middle). On a cylinder with a small diameter (high curvature), septins align parallel to the cylinder main axis, whereas on a cylinder with a larger diameter, they arrange orthogonally to its axis. Reproduced from Cannon et al., 2019 (© 2019 Cannon et al.; published under CC-BY 4.0 license). Right: Binding of septins to a lipid bilayer deposited on a wavy substrate presenting various curvatures (here, maximum curvature is $+ \text{ or } - 3 \mu\text{m}^{-1}$). At regions of negative curvature, septins bend negatively and thus follow the substrate curvature, whereas they remain straight when interacting with positively curved regions, *i.e.* at the top of the “hills” and parallel to the ridges of those hills. Reproduced from (Beber et al., 2019b). (D and E) Two models have been proposed to explain the curvature preference of septins. (D) Curvature sensing might result from the insertion of an amphipathic helix (green

cylinder) of the septin filament (thick green line) into defects of a curved lipid bilayer. (E) Geometric consideration underlying the curvature preference of septins: to maximize the area of interaction with the membrane, the filaments would need to bend. The red solid line represents the contact surface between the septin filament and the bilayer. The blue solid line represents the opposite side of the filament. The surface of contact is higher when the membrane contacts septins on a concave curvature.

Fig. 4 Septins can deform liposomes. A. A GUV is shown that has been incubated with cell extracts containing human septins and other proteins and factors, resulting in membrane tubulations. Reproduced with permission from (Tanaka-Takiguchi et al., 2009), B. GUVs incubated with purified budding yeast septins exhibit periodic spiky deformations. Reproduced from (from Beber et al., 2019b).

Acknowledgements

We thank Patricia Bassereau and Daniel Lévy for useful advice and discussions. This work benefited from the support of the ANR (Agence Nationale de la Recherche) for funding the project "SEPTIME", ANR-13-JSV8-0002-01 and the project "SEPTSCORT", ANR-20-CE11-0014-01. B. Chauvin is funded by the Ecole Doctorale "ED564: Physique en Ile de France" and Fondation pour la Recherche Médicale. K. Nakazawa was supported by Sorbonne Université (AAP Emergence). We thank the Labex Cell(n)Scale (ANR-11-LABX0038) and to Paris Sciences et Lettres (ANR-10-IDEX-0001-02). We thank the Cell and Tissue Imaging (PICT-IBiSA), Institut Curie, member of the French National Research Infrastructure France-BioImaging (ANR10-INBS-04).

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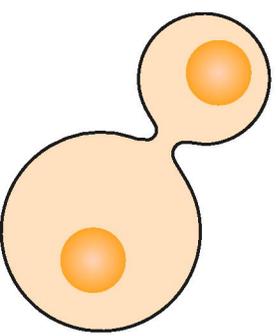
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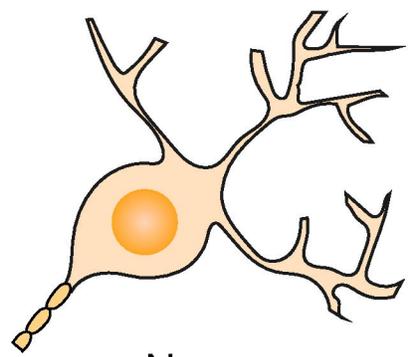
Septin location *in vivo*

Division neck



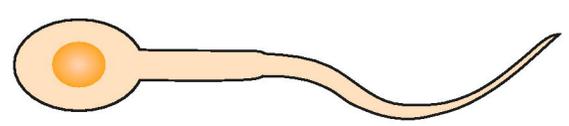
Yeast cell

Base of dendritic protrusion,
blanching point



Neuron

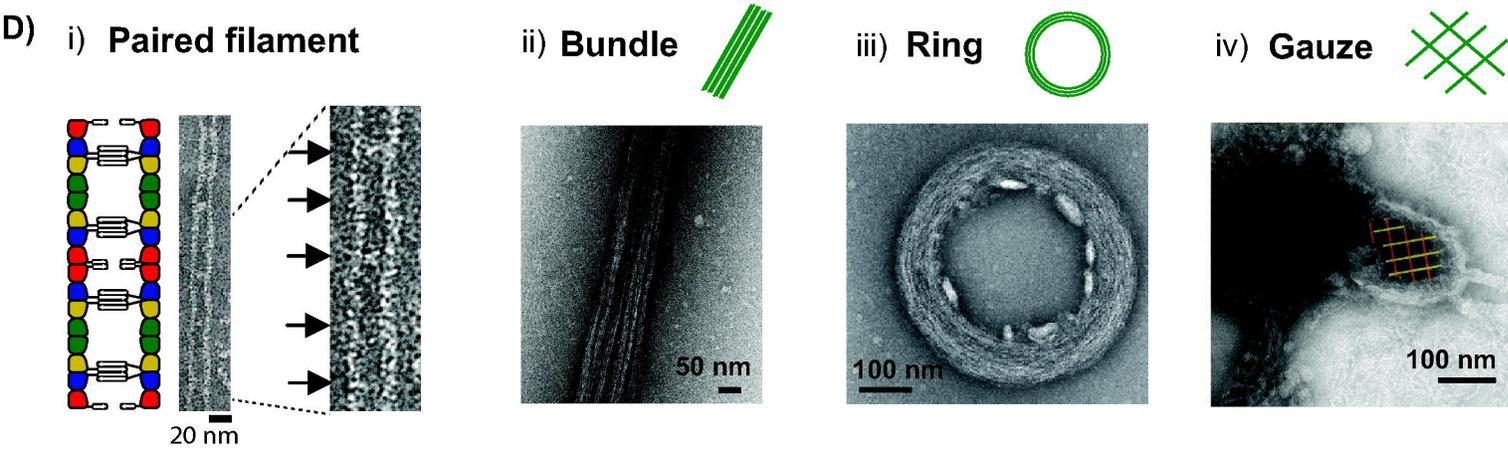
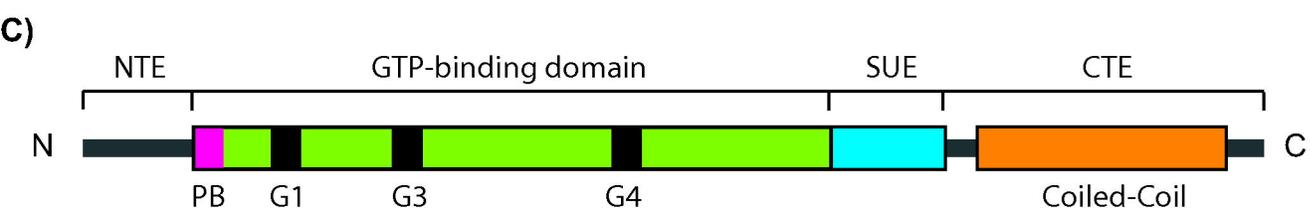
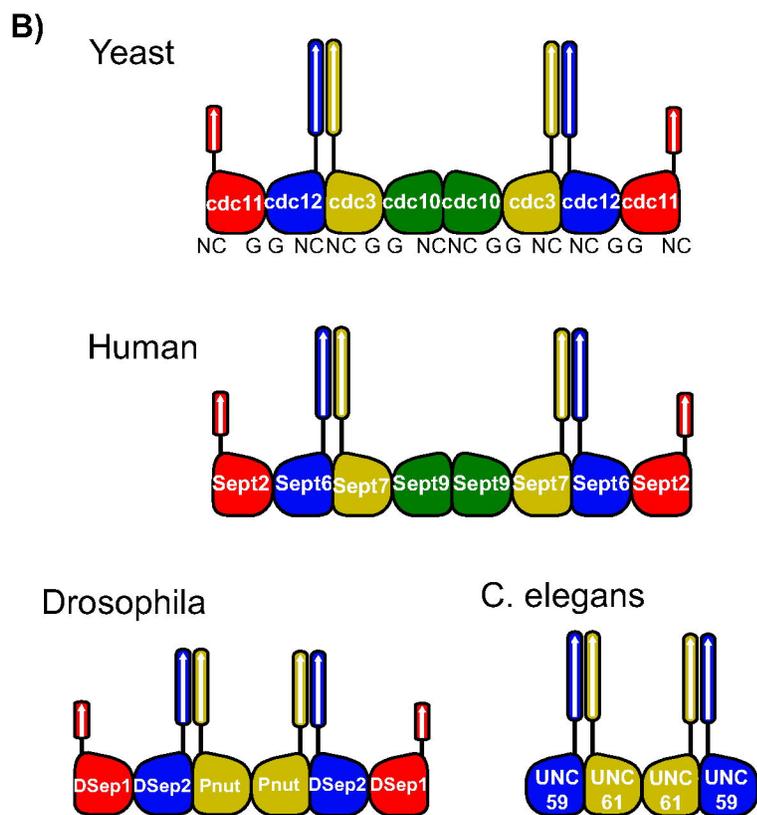
Annulus

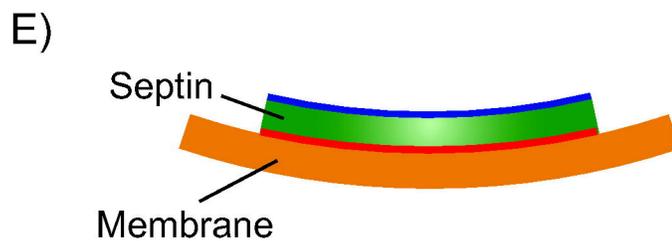
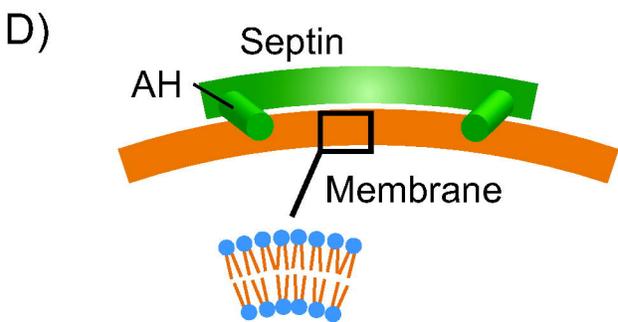
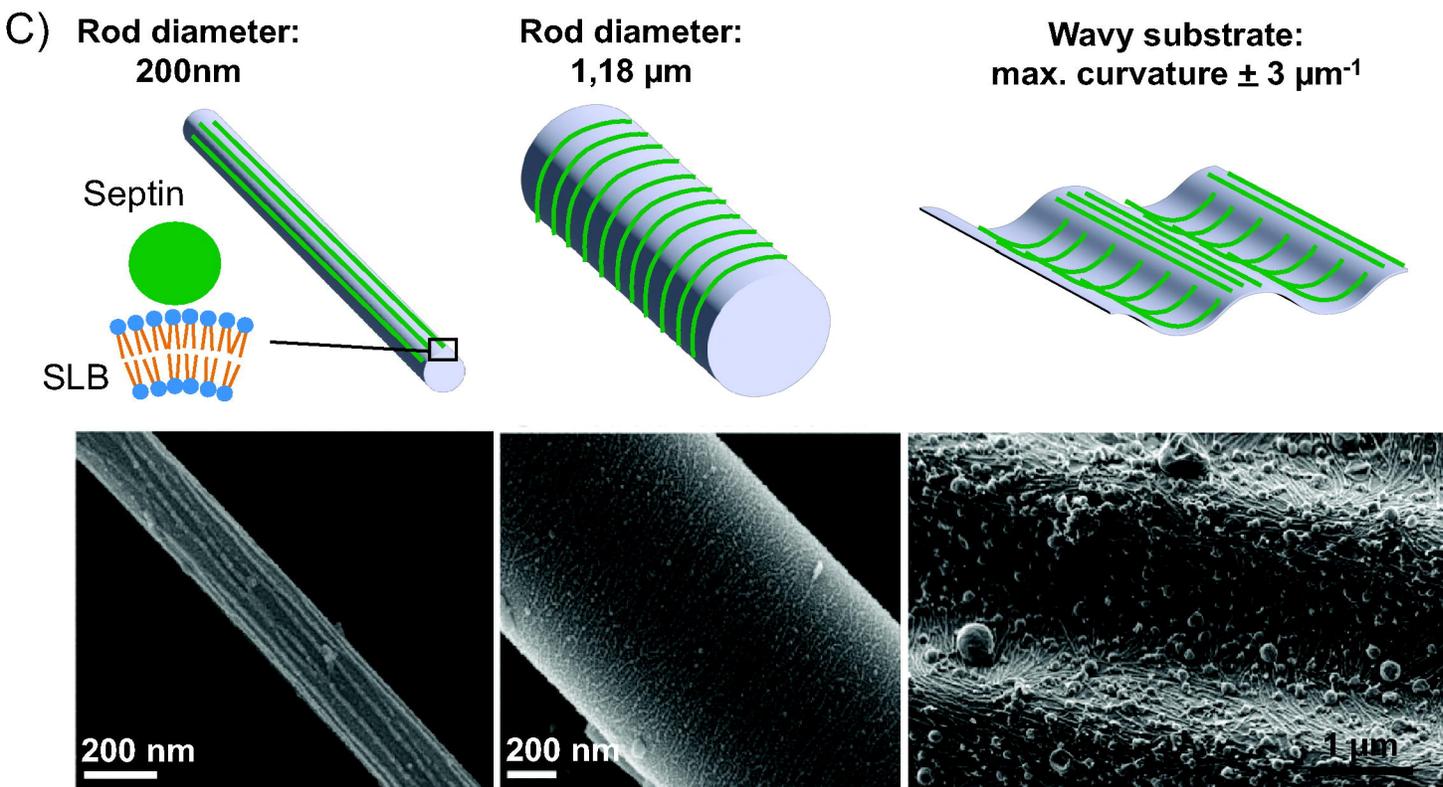
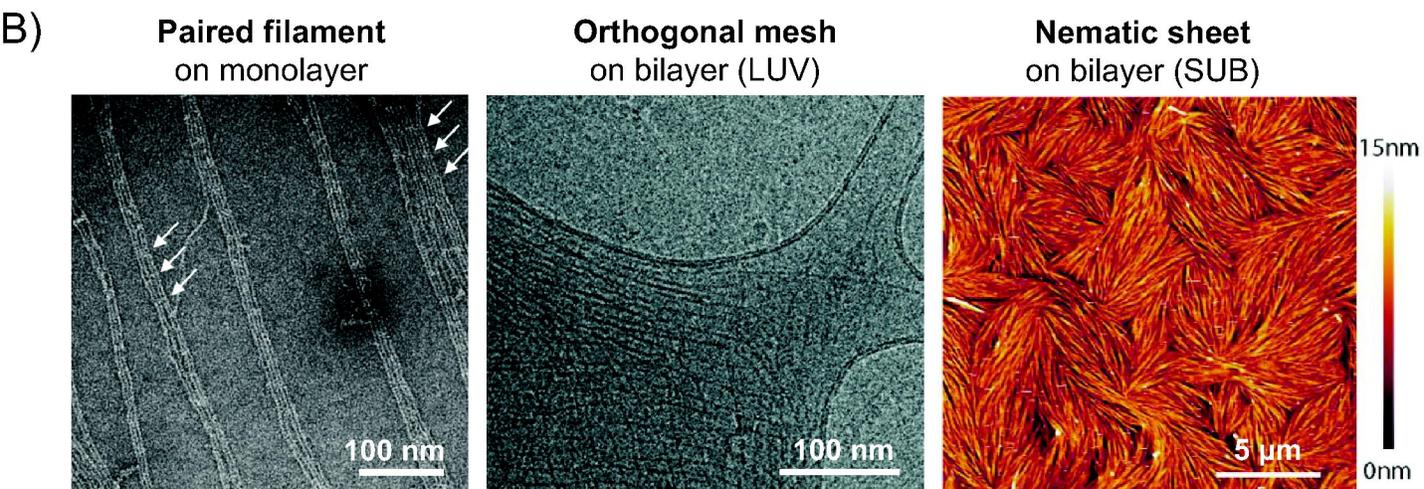
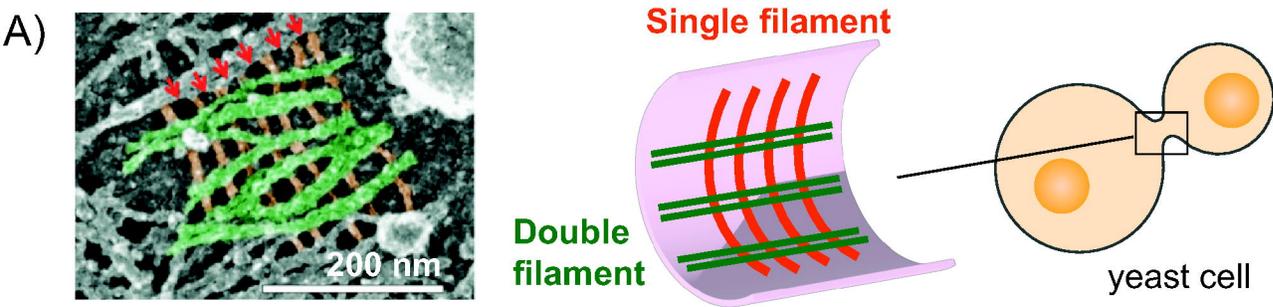


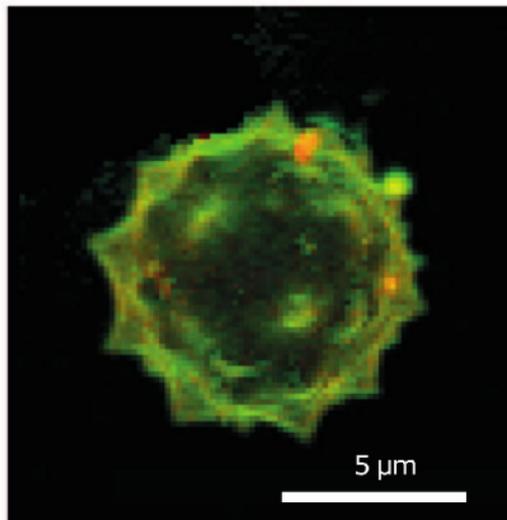
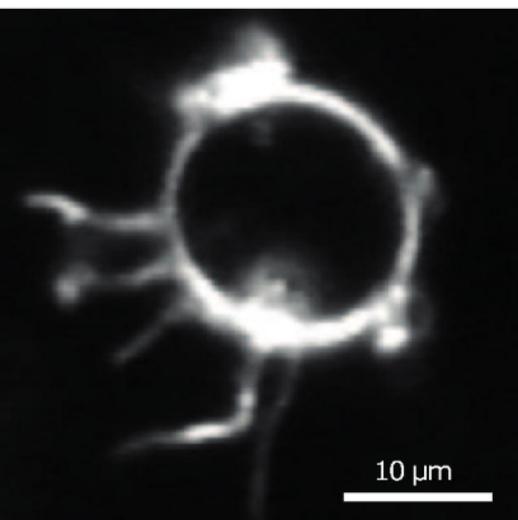
Spermatozoid

A)

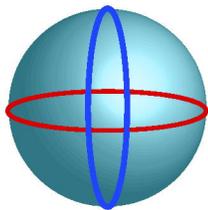
Momany Group	Kinoshita Group	Hs	Dm	Ce	Sc
1a	SEPT3	SEPT3 SEPT9 SEPT12			Cdc10
1b	SEPT6	SEPT6 SEPT8 SEPT10 SEPT11 SEPT14	Sep2 Sep5	UNC61	
2a					Cdc3
2b	SEPT2	SEPT1 SEPT2 SEPT4 SEPT5	Sep1 Sep4	UNC59	
	SEPT7	SEPT7	Pnut		
3					Cdc11 Shs1
4					Cdc12







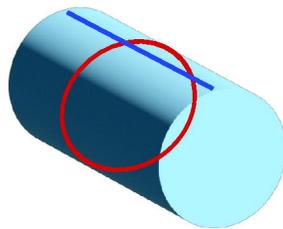
$R_1 > 0$ $R_2 > 0$



Gaussian
Curvature K
($K = k_1 * k_2$)

$K > 0$

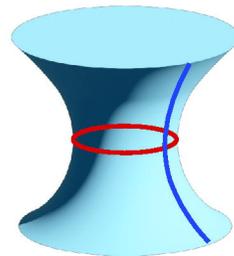
$R_1 > 0$ $R_2 = 0$



Mean
Curvature H
($H = (k_1 + k_2) / 2$)

$K = 0$

$R_1 > 0$ $R_2 < 0$



$K < 0$

$H = 0$ (if $R_1 = R_2$)

