

# UC Berkeley

## UC Berkeley Previously Published Works

### Title

Turning it inside out: The organization of human septin heterooligomers.

### Permalink

<https://escholarship.org/uc/item/5m3906k3>

### Journal

Cytoskeleton, 76(9-10)

### Authors

McMurray, Michael  
Thorner, Jeremy

### Publication Date

2019-09-01

### DOI

10.1002/cm.21571

Peer reviewed



# HHS Public Access

Author manuscript

*Cytoskeleton (Hoboken)*. Author manuscript; available in PMC 2019 November 22.

Published in final edited form as:

*Cytoskeleton (Hoboken)*. 2019 September ; 76(9-10): 449–456. doi:10.1002/cm.21571.

## Turning it inside out: the organization of human septin hetero-oligomers

Michael A. McMurray<sup>1</sup>, Jeremy Thorner<sup>2</sup>

<sup>1</sup>Department of Cell and Developmental Biology, University of Colorado Anschutz Medical Campus, Aurora, CO 80045 USA

<sup>2</sup>Division of Biochemistry, Biophysics & Structural Biology and Division of Cell & Developmental Biology, Department of Molecular and Cell Biology, University of California, Berkeley, CA 94720 USA

### Abstract

Septin family proteins are quite similar to each other both within and between eukaryotic species. Typically, multiple discrete septins co-assemble into linear hetero-oligomers (usually hexameric or octameric rods) with a variety of cellular functions. We know little about how incorporation of different septins confers different properties to such complexes. This issue is especially acute in human cells where 13 separate septin gene products (often produced in multiple forms arising from alternative start codons and differential splicing) are expressed in a tissue-specific manner. Based on sequence alignments and phylogenetic criteria, human septins fall into four distinct groups predictive of their interactions, *i.e.* members of the same group appear to occupy the same position within oligomeric septin protomers, which are “palindromic” (have two-fold rotational symmetry about a central homodimeric pair). Many such protomers are capable of end-to-end polymerization, generating filaments. Over a decade ago, a study using X-ray crystallography and single-particle electron microscopy deduced the arrangement within recombinant hetero-hexamers comprising representatives of three human septin groups— SEPT2, SEPT6, and SEPT7. This model greatly influenced subsequent studies of human and other septin complexes, including how incorporating a septin from a fourth group forms hetero-octamers, as first observed in budding yeast. Two recent studies, including one in this issue of *Cytoskeleton*, provide clear evidence that, in fact, the organization of subunits within human septin hetero-hexamers and -octamers is inverted relative to the original model. These findings are discussed here in a broader context, including possible causes for the initial confusion.

### Keywords

septins; protein structure; crystallography; X-ray; microscopy; electron; cytoskeleton; polymerization; oligomerization

### The septin protein family

Septins exist in nearly all eukaryotes and are GTP-binding proteins. In most organisms, they are found in filamentous structures containing other septins. It has been proposed that septins evolved from a primordial homodimeric GTPase (Weirich, Erzberger & Barral,

2008), and their close structural relative Toc34 (a nuclear-encoded plant protein involved in translocation of precursor proteins into chloroplasts) forms a homodimer (Wiesemann *et al.*, 2019). Unlike Toc34, septin gene expansion occurred during evolution to the point that, for example, mammals have more than 10 and even the unicellular eukaryote budding yeast has seven.

In humans, each of 13 septin genes encodes a protein with >35% sequence identity to the products of any of the other genes. Due to differential splicing and alternative translational start sites, certain human septins harbor additional sequences not shared with the other septins or, in some cases, lack sequences common to other septins. Nonetheless, for the most part, they look quite alike and this clear resemblance makes it relatively easy to recognize a protein as a septin (Pan, Malmberg, & Momany, 2007). In keeping with their proposed evolutionary origin, many septins have been observed to self-associate (Mendoza, Hyman, & Glotzer, 2002; Serrão *et al.*, 2011; Zent, Vetter, & Wittinghofer, 2011; Pissuti Damalio *et al.*, 2012). Given these properties, how do such similar proteins hetero-oligomerize, and how is each subunit directed to occupy a defined position within the hetero-oligomeric complex?

These questions are among the oldest quandaries in the septin field. However, in 2007, it seemed that a solution was at hand when Sirajuddin *et al.* (2007) reported analysis of purified recombinant septin complexes composed of three human septins (SEPT2, SEPT6 and SEPT7), which are reflective of native septin complexes that can be isolated from cultured mammalian cells (Kinoshita *et al.*, 2002; Nagata *et al.*, 2004). From examination of crystals of these complexes (as well as crystals of SEPT2 alone) by X-ray diffraction and viewing individual complexes by transmission electron microscopy (EM), Sirajuddin *et al.* determined that the complex was a linear hetero-hexamer and proposed a model for the position of each of the three human septins in the hetero-hexamer (Sirajuddin *et al.*, 2007) (Figure 1).

## Challenges in determining the subunit arrangement in septin hetero-oligomers

It is instructive to consider the rationale behind and the caveats inherent within the experiments carried out by Sirajuddin *et al.* (2007) to derive their hugely influential model. Several experimental approaches allow an investigator to determine which proteins interact directly with each other in a multi-subunit complex. For septins, one approach involves purifying each individual septin independently as a recombinant protein in a host that lacks endogenous septins (*e.g.*, *Escherichia coli*) and then examining whether, when mixed together pair-wise or in even greater combinations, interactions among them can be readily detected *in vitro* by standard biochemical methods (co-immunoprecipitation, size exclusion chromatography, glycerol gradient sedimentation, etc.). The assumption is that only septins that directly contact each other in the context of a native septin complex will interact with high affinity when they encounter each other in purified form. However, instability, misfolding and aggregation of individually-expressed full-length septins has often been a problem (Hu *et al.*, 2006; Garcia *et al.*, 2007; Pissuti Damalio *et al.*, 2012; Khairat *et al.*, 2017), and even more confounding is the previously mentioned tendency of individual

septins obtained in this way to associate as homodimers. If a septin normally homodimerizes in its native context, then its ability to homodimerize during or after synthesis in a heterologous host is a valuable clue. On the other hand, if a septin in its native context normally hetero-dimerizes with a different septin, and formation of such a heterodimer interface is impossible because the natural partner is missing, then homodimerization of a septin in this situation could be a red herring. Indeed, preparations of human SEPT2, SEPT6, or SEPT7 that were each expressed individually in and purified from *E. coli* were all mixtures of monomers and homodimers (Low & Macara, 2006). Similarly, understanding of septin oligomer formation was further complicated by an earlier report that, when purified individually, the SEPT2 homolog from the frog *Xenopus laevis* was able to assemble into homopolymeric filaments *in vitro* (Mendoza, Hyman & Glotzer, 2002), raising the possibility at the time that, in the cell too, such homomeric septin oligomers and filaments might exist. We now know, however, that although promiscuous septin interactions can be observed when a particular class is omitted, a full complement of each distinct septin type always forms the same hetero-oligomeric complex with the corresponding subunits in an invariant order.

In principle, one way to avoid non-native homodimerization is to express two or more human septins simultaneously in the same host cells, which presumably provides the normal native partner(s) and precludes promiscuous interactions. Indeed, co-expression of SEPT2, SEPT6 and SEPT7 in *E. coli* (Sheffield *et al.*, 2003; Sirajuddin *et al.*, 2007) or in insect cells via baculovirus infection (Kinoshita *et al.*, 2002) results in co-purification of a complex containing a 1:1:1 ratio of each septin component and with biophysical properties and appearance in single-particle EM consistent with a linear, rod-shaped hexamer. However, these findings alone do not reveal the organization of septins within the rod. A seemingly obvious means to that end is simply to omit one of the septins from the co-expression system; whether and how many of the remaining septins associate might, in theory, provide some indication of where the chain was broken and thus which link was missing. However, when pairwise combinations of SEPT2, SEPT6, and SEPT7 were co-expressed, the two co-expressed septins co-purified as hetero-dimers in every case (Sheffield *et al.*, 2003), a result incompatible with a linear subunit arrangement in the hetero-hexameric complex. So, once again, we are confronted with the same problem, epitomized lyrically in the chorus of the Stephen Stills song— “If you can’t be with the one you love, then love the one you’re with.”

There are, however, two techniques that can reveal by EM where an individual subunit resides within a septin hetero-oligomer. First, if there is available an antibody uniquely specific for the septin of interest (*i.e.*, non-cross-reactive against any other septin present in the mixture), one can decorate the complexes with such an antibody and examine the resulting particles. The “Y” shape of an antibody molecule is fairly easy to recognize by negative staining and the forked tip of the “Y” should contact the target septin in the rod. This antibody-labeling approach established, for example, that Cdc11 occupies the terminal position at each end of the hetero-octameric septin complex from budding yeast (Bertin *et al.*, 2008). However, this approach was not used by Sirajuddin *et al.* in the analysis of their human septin complexes, perhaps because appropriate antibodies were not available. Of course, because their complexes were generated by recombinant expression, they could have

ected to install short epitope tags for which highly selective and high-affinity monoclonal antibodies are widely available to achieve the same end, but they did not.

A second method for subunit identification within recombinant septin complexes is to fuse the septin of interest to a small protein tag (*e.g.*, GFP) large enough to provide, by itself, extra juxtaposed density in the negative-stained images and, thus, to serve as a fiducial marker to register the location of that subunit. Of course, it must be documented that installation of such a tag does not comprise either function or formation of the complex. Indeed, Sirajuddin *et al.* elected to use this approach; they fused the *E. coli MalE* gene product (maltose-binding protein; MBP) to the N terminus of full-length SEPT2 (361 residues) and co-expressed this chimera with SEPT6 (434 residues) and SEPT7 (437 residues) in bacterial cells from which they purified the resulting complexes (Sirajuddin *et al.*, 2007). MBP (370 residues) is a nearly spherical protein quite similar in size and shape to the globular GTPase domain of SEPT2. Hence, in addition to the six “blobs” already observed under EM for the hexameric SEPT2-, SEPT6- and SEPT7-containing complex, in the complex composed of MBP-SEPT2, SEPT6 and SEPT7, ideally, there should be a “new” blob adjacent to the position of SEPT2. Indeed, additional blobs were seen, and they seemed to be near the centers of the hexamers (Sirajuddin *et al.*, 2007). When they examined the crystal structure of a C-terminally truncated version of SEPT2, they observed that SEPT2( 316–361) self-associated in chains via two alternating interaction modes: one where the guanine nucleotide-binding pockets face each other (dubbed the G-interface) and the other approximately 180° away where elements N-terminal to the GTPase domain and elements C-terminal to the GTPase domain interact (dubbed the NC-interface). At this time, they did not perform similar studies on the GTPase domains of either SEPT6 or SEPT7. In any event, on the basis of their observations on the crystals of SEPT2( 316–361), it seems understandable why it may have been assumed that in the hexamers too, SEPT2 might be present as a homodimer. Moreover, because they thought they saw extra blobs at the center of the rods in their MBP-SEPT2-, SEPT6- and SEPT7-containing complexes, they presumed that a SEPT2-SEPT2 dimer must be present at the center of the hetero-hexamer.

A typical and often necessary step in the structural analysis of large complexes by EM is to take the images of many separate particles that closely resemble each other and average them together, which blurs out the non-specific background and enhances the resolution of the real densities present in such “class averages.” We note that in the class averages of the MBP-SEPT2-, SEPT6- and SEPT7-containing complexes reported by Sirajuddin *et al.* (2007), two new blobs were not seen at the center of the rod at the putative location of SEPT2, but this is not necessarily surprising. Although the N-terminal sequence upstream of the GTPase domain of SEPT2 is one of the shortest among human septins, it nonetheless projects away from the globular domain. Hence, the MBP tag will be separated from the SEPT2 globular domain by the length of the N-terminal sequence of SEPT2 and whatever additional sequence was appended to the C-terminus of MBP to link it to SEPT2 [unfortunately, the necessary details are not available either in Sirajuddin *et al.* (2007) or in Sirajuddin’s Ph.D. thesis]. If the fiducial tag is attached by a tether that is too flexible, in any given particle, the tag near one SEPT2 may not be in the same orientation as the tag near the other copy of SEPT2 and, hence, only one of the two may appear in any given class average. Indeed, a similar situation was observed when EM was used to examine the organization of

subunits within a tetrameric two-septin complex from the nematode *Caenorhabditis elegans* in which GFP was appended to the C-terminus of one of the septins; in the class averages, the resulting blob was found in a range of positions, describing an arc of a fixed distance from the location of the septin to which it was fused (John *et al.*, 2007). In the work by Sirajuddin *et al.* (2007), only a single image of just one un-averaged hexamer was provided in the which there appeared to be two extra densities near the middle of a hexameric rod.

When one approach supports a particular model, but is not in itself conclusive, caution demands a second independent approach; if the two results agree, the findings overall are more convincing. In protein crystallography, one way to install the equivalent of fiducial marks to inform structure determination (*i.e.*, solve the phase problem) is via replacement of methionine in the protein of interest with selenomethionine. Because the atomic mass of selenium is 2.5 times that of sulfur it scatters more X-rays, thereby revealing the location of these residues within the density map. Sirajuddin *et al.* used this approach to solve the crystal structure of their SEPT2-, SEPT6- and SEPT7-containing complexes to about 4Å resolution (Sirajuddin *et al.*, 2007). However, the asymmetric unit in their crystals was a trimer, not a hexamer. Therefore, although their X-ray diffraction analysis yielded the unambiguous order SEPT2-SEPT6-SEPT7 in these trimers, it could not unequivocally pin down how trimer-trimer association yields the full hexameric complex (Figure 1A). The reason for the uncertainty, and the special challenge of septin crystallography, is that the septin complexes in these crystals are arranged in polymeric orientation and it is not necessarily the case that how polymerization occurs under native conditions in solution will be reflected in the arrangement observed in the crystal. Indeed, Sirajuddin *et al.* assumed that the trimer-trimer contacts observed in the crystal (via SEPT7-SEPT7 association) were indicative of the mechanism of polymerization in solution. By combining this assumption with the single-particle EM data using the MBP tag as a fiducial mark for SEPT2, the authors concluded that the arrangement of human septins in the hetero-hexamer is SEPT7-SEPT6-SEPT2-SEPT2-SEPT6-SEPT7 (Sirajuddin *et al.*, 2007) (Figure 1B). As we explain below, other data, particularly those from the work of Mendonça *et al.* in this issue of *Cytoskeleton*, indicate rather unequivocally that, in fact, the order of subunits in the human hetero-hexamer is inverted from that proffered by Sirajuddin *et al.*, namely SEPT2-SEPT6-SEPT7-SEPT7-SEPT6-SEPT2 (see Figure 1D).

### Hints from subsequent studies that perhaps something was amiss

The work of Sirajuddin *et al.* (2007) made a big splash in the septin field. In a meeting report about the conference at which these results were first presented prior to publication, Gladfelter & Montagna (2007) lamented how “many of us had to say farewell to our favourite models of septin organization.” However, there were troublesome clues that the original model of human hexamer organization was not quite right. The first concern was a peculiar feature of the hexamers Sirajuddin *et al.* saw by EM. First, Sirajuddin *et al.* (2007) stated in their Results that half the hexamers in their preparations of “native” SEPT2-, SEPT6- and SEPT7- containing complexes exhibited a noticeable “kink” [see Fig. 3f in Sirajuddin *et al.* (2007)] and half did not. This observation already provided a hint that, even before they appended MBP to the N terminus of SEPT2, something was off. Second, their preparations of MBP-SEPT2-, SEPT6- and SEPT7- containing complexes were even more

markedly kinked in the middle [see Fig. 3c in Sirajuddin *et al.* (2007)]. By contrast, no kinks were observed in yeast septin rods examined under the same conditions [see Fig. 3e in Sirajuddin *et al.* (2007)] or by others (Bertin *et al.*, 2008). Likewise, native septin hexamers purified from rat brain were not kinked in 3D reconstructions generated from single-particle EM images (Lukoyanova, Baldwin & Trinick, 2008). Unfortunately, the order of the subunits in the rat rods was ambiguous due to the lack of fiducial markers. Moreover, the actual subunit composition of these rat complexes is uncertain because not all the constituent proteins were accounted for. Hence, the conclusion that the hexameric particles examined were composed solely of the rat septins Sept3, Sept5, and Sept7 (Lukoyanova, Baldwin & Trinick, 2008) remains questionable and would represent an arrangement incompatible with contemporary understanding of mammalian septin hetero-oligomer formation because a representative of the SEPT6 group was purportedly lacking. In any event, the kinks in the SEPT2-, SEPT6- and SEPT7-containing hexamers observed by Sirajuddin *et al.* appear anomalous.

A second clue came from subsequent studies of representatives of the fourth major subgroup in the human septin family (SEPT3, SEPT9 and SEPT12). Specifically, when SEPT9 was present, it interacted directly with SEPT7 (Kim *et al.* 2011). Kim *et al.* also examined the effect on hexamer formation of mutations designed to destabilize the SEPT6–SEPT7 NC interface, which confirmed that SEPT6 is situated between SEPT2 and SEPT7 (Kim *et al.*, 2011). In addition to fitting with the existing hexamer model, it was probably conceptually easiest to imagine hexamers becoming octamers simply by appending one additional subunit at each end. So, assuming that the model from Sirajuddin *et al.* was correct, collectively these findings suggested that SEPT9 must occupy the terminal positions within human septin hetero-octamers (Figure 1C), and others reached the same conclusion for similar reasons for human SEPT9 and other SEPT3 group members (Sellin *et al.*, 2011; Sellin, Stenmark, & Gullberg, 2014). However, the SEPT3 group septins are more closely related to budding yeast Cdc10 than to any of the other yeast septins (Pan, Malmberg & Momany, 2007), and it was demonstrated convincingly that a Cdc10-Cdc10 NC homodimer resides at the center of the yeast septin hetero-octamers, not at its ends (Bertin *et al.*, 2008) (see Figure 1F). Furthermore, in the absence of Cdc10, yeast cells can assemble non-native hexamers in which the central pair is a Cdc3-Cdc3 G homodimer (McMurray *et al.*, 2011); some filamentous fungi, such as *Aspergillus nidulans*, seem to make such hexamers, in addition to octamers, during the normal course of their development (Hernández-Rodríguez *et al.*, 2014). So, why would human septins have evolved any differently?

## Evidence demanding a revised order of subunits in human septin hetero-oligomers

Hints from the literature notwithstanding, it is fair to say that until very recently the septin field accepted the arrangement of the septins within human hetero-hexamers as proposed by Sirajuddin *et al.* (2007) and within hetero-octamers as proposed by Kim *et al.* (2011). Everything changed with the deposition on the same day in March 2019 of two preprints in the bioRxiv (Mendonça *et al.*, 2019; Soroor *et al.*, 2019). One study, a revised version of which is now been published in this issue of *Cytoskeleton*, uses antibody decoration and



MBP-tagged subunits of two different representatives of the SEPT2 group, SEPT2 itself and SEPT5, to provide compelling support for the conclusion that the order of subunits in these human septin hetero-hexamers is SEPT2 (or SEPT5)-SEPT6-SEPT7-SEPT7-SEPT6-SEPT2 (or SEPT5) (Figure 1D), an order that is the exact inverse of that deduced by Sirajuddin *et al.* (2007). The other study is complementary because it uses primarily *in vivo* evidence to arrive independently at the same conclusion and, further, provides evidence that a SEPT9-SEPT9 NC homodimer occupies the central location in human septin hetero-octamers.

Mendonça *et al.* (2019) set out to purify a complex containing human SEPT5, SEPT6, and SEPT7 following heterologous co-expression in *E. coli*. SEPT5 is closely related to SEPT2 (Pan, Malmberg & Momany, 2007), hence the authors expected to purify hexamers containing a 1:1:1 ratio of these three proteins and, indeed, they did. However, when they examined otherwise identical complexes containing MBP-SEPT5 (instead of the untagged version) by single-particle EM, the extra blobs in their images were very clearly at one or both ends of the rod, not the middle, despite the linkers needed to join MBP to SEPT5. To corroborate the conclusion that SEPT5 was located at the terminus of each hexameric rod, they incubated their native preparations with anti-SEPT5 antibodies and found in their EM images that the antibody clearly decorated only the ends of rods, providing further evidence that SEPT5 occupies the two terminal positions in the hetero-hexamers.

SEPT2 shares 63% identity and 80% similarity with SEPT5, but perhaps even modest differences between them could drive the two septins to occupy different positions within a hexamer. To address this possibility, Mendonça *et al.* directly repeated the experiments of Sirajuddin *et al.*, preparing hetero-hexamers containing MBP-SEPT2, SEPT6 and SEPT7, and, in their hands of these investigators, the hexameric rods were uninked and the blobs of MBP density were very clearly juxtaposed to the end, not the middle, of the rod. Thus, MBP-SEPT2 behaved just like MBP-SEPT5.

In the independent work by Soroor *et al.*, an entirely independent path was taken to the same conclusion. They analyzed the oligomeric state of septin complexes purified from human cells in which they manipulated the levels of SEPT9 or mutants thereof (Soroor *et al.*, 2019). One such mutant blocks SEPT9-SEPT9 NC homodimerization. Polymerization *in vitro* of purified septin complexes into filaments is sensitive to the ionic strength of the solution, with filaments falling apart at salt concentrations above those that are considered physiological. The original model predicted that the SEPT9 NC interface mutant should “cap” the ends of octamers and prevent their polymerization into filaments in low/physiological salt, while still having the capacity to form octamers in high salt (Figure 1C). This SEPT9 mutant blocked filament formation *in vivo*, as expected, but in high salt the mutant generated tetramers (Soroor *et al.*, 2019), just like yeast complexes with the equivalent mutation in Cdc10 (Bertin *et al.*, 2010; McMurray *et al.*, 2011) (Figure 1F). Analogous experiments with a homodimerization-incompetent mutant of SEPT2 resulted not in high-salt tetramers and trimers, as predicted by the original model (Figure 1C), but in octamers and hexamers (Soroor *et al.*, 2019). Finally, the original model predicts that hexamers and octamers should be unable to co-polymerize in low salt, because they differ in which septin-septin interface is exposed at their termini (Figure 1B and C). On the contrary, purified hexamers and octamers readily co-polymerized *in vitro*. These results strongly support an order of subunits in the



human hetero-octamers of SEPT2–SEPT6–SEPT7–SEPT9–SEPT9–SEPT7–SEPT6–SEPT2 (Figure 1E). Thus, the overall organization of the human hetero-octamer now is essentially congruent with that determined for yeast septin hetero-octamers (Bertin *et al.*, 2008) (Figure 1E and F).

### Working out the kinks: possible sources of confusion

To attempt to rationalize the discrepancy between their results and those of Sirajuddin *et al.* (2007), Mendonça *et al.* noted that technological advances in EM likely improved their ability to visualize the extra density in the complexes containing MBP-SEPT2 and MBP-SEPT5. Soroor *et al.* speculated, instead, that “it is possible that the two trimeric halves [of the hexamer] came together in an unconventional way with SEPT7 at the ends and therefore presented a model of an artifactual complex” (Soroor *et al.*, 2019). We favor the latter explanation, and here we identify possible sources of such artifacts.

Reminiscent of the kinks in the human hexamers described by Sirajuddin *et al.* (2007), we saw kinks in yeast hexamers formed by a Cdc3-Cdc3 G homodimer interface in the absence of Cdc10 (McMurray *et al.*, 2011) and in yeast octamers containing Cdc10 mutations that weaken, but do not completely cripple, its NC homodimerization interface (Bertin *et al.*, 2010). Therefore, it seems reasonable to propose that the hexamers visualized in Sirajuddin *et al.* were held together by an unusually weak, flexible connection. Intrigued by this possibility, we noted in the Methods section of the Sirajuddin *et al.* (2007) study that they found it necessary to stabilize their hexamers via pre-fixation with glutaraldehyde before they examined their complexes by EM. Such a treatment was not needed in any other single-particle EM study of other septin complexes, including those of Mendonça *et al.* (2019). What could have destabilized the hexamers of Sirajuddin *et al.*?

We also note that Sirajuddin *et al.* (2007) prepared their MBP-SEPT2-containing complexes differently than they did the untagged complexes: “Because complex formation capacity in the MBP–SEPT2 fusion complex is reduced, centrifugation and fixation at slightly lowered salt was necessary to obtain stable hexameric septin complexes. High salt conditions without gradient centrifugation and chemical stabilization did result in fragmentation of septin complexes containing the MBP–SEPT2 fusion complex. Lower salt conditions without gradient centrifugation and chemical stabilization, on the other hand, yielded a highly heterogeneous complex population (data not shown).” Salt concentration and septin-septin interaction have a complicated relationship. The very first septin crystal structure, also reported in Sirajuddin *et al.*, was that of SEPT2 (or, at least, a version lacking one amino acid from the N terminus and 56 from the C terminus) expressed and purified individually (Sirajuddin *et al.*, 2007). As with full-length SEPT2, the individual truncated septin behaved as a mix of monomers and homodimers; within the asymmetric unit of the crystal, SEPT2 homodimerized via both its G and NC interfaces (Sirajuddin *et al.*, 2007). By mutating key residues that they saw made specific contacts across one or the other interface, Sirajuddin *et al.* were able to determine that, in solution, SEPT2 homodimers formed preferentially via their G interface (Sirajuddin *et al.*, 2007). In their hexamer model, however, where they placed a pair of SEPT2 subunits at the center, the interaction between them was predicted to be mediated via an NC interface. A key experiment that could have reconciled this

discrepancy would have been to examine complexes produced upon co-expression of SEPT6 and SEPT7 with SEPT2 carrying either of the mutations they designed to disrupt SEPT2 NC homodimerization. According to the hexamer model from Sirajuddin *et al.* (Figure 1B) the NC-disrupting SEPT2 mutation would have led to the formation of trimers. Instead, Sirajuddin *et al.* chalked up the observation of G homodimerization by isolated SEPT2 G as “love the one you’re with”, and speculated that the reason hexamer polymerization into filaments is salt-sensitive is because the SEPT7–SEPT7 G homodimer is salt-sensitive (Sirajuddin *et al.*, 2007). Considering that subsequent work in budding yeast established that it is the high-salt sensitivity of an NC homodimer interface that results in disassembly of yeast septin filaments into its constituent hetero-octameric protomers (Bertin *et al.*, 2008) (Figure 1F), the fact that SEPT2 forms a G homodimer rather than an NC homodimer in solution may say more about the physiological properties of the SEPT2 NC homodimer interface (i.e., that it is salt-sensitive) than the ability of SEPT2 to undergo non-physiological self-association via a G interface. These considerations, in retrospect, are at least consistent with SEPT2 occupying a position at the ends, rather than the middle, of human septin hetero-oligomers, as we now know is the case.

It is not obvious from any model of septin arrangement why fusing MBP to the N terminus of SEPT2 would have destabilized hexamers in the hands of Sirajuddin *et al.* (2007), but the revised model predicts that the SEPT2 NC homodimer is salt-sensitive (Figure 1D). Indeed, Mendonça *et al.* (2019) have applied the Proteins Interfaces Structures and Assemblies (PISA) analysis tool to predict the salt sensitivity of the SEPT2 NC homodimer interface compared to that of the SEPT7 G homodimer, and found that the SEPT2 NC homodimer should be much more sensitive to salt. Lowering the salt would therefore be expected to promote SEPT2 NC homodimerization.

In a search for other clues, we examined the additional data available in the Ph.D. thesis of Sirajuddin (2007). SDS-PAGE profiles available therein clearly document that mainly a C-terminally truncated form of SEPT7 was present in the purportedly native complexes (and this proteolysis was 100% complete in the crystals they redissolved and analyzed), which likely destabilized the already suboptimal SEPT7-SEPT7 G interface. Detectable truncated SEPT7 is also present in the complexes analyzed by Mendonça *et al.*, but to a much lesser extent. Sirajuddin *et al.* showed that deliberately removing the extended C termini of all three septins did not prevent them from being able to isolate hexameric SEPT2-, SEPT6-, and SEPT7-containing complexes (Sirajuddin *et al.*, 2007). Nonetheless, we strongly suspect that the unwanted loss of C-terminal sequences from SEPT7 destabilized those hexamers because removal of C-terminal sequences from two yeast septins Cdc3 and Cdc12 clearly destabilized hetero-octamers compared to those generated from their full-length counterparts (Bertin *et al.*, 2010). We presume that adding MBP to SEPT2 further destabilized the human hexamer (for some reason) to the point that the hexamers fell apart completely into trimers, unless, as Sirajuddin *et al.* (2007) stated, the salt concentration was lowered, presumably favoring MBP-SEPT2-MBP-SEPT2 NC association over SEPT7-SEPT7 homodimerization, which they further stabilized by glutaraldehyde fixation. Alternatively, if the tether connecting the C terminus of MBP to the N terminus of SEPT2 was sufficiently long and floppy, it is even possible that the glutaraldehyde treatment affixed MBP to a particularly exposed or reactive residue in SEPT7, such that even an intact hexamer with SEPT2 at its

ends would appear to have an MBP blob(s) at its center. Either of these scenarios could have conspired to yield the “inside-out” hexamer observed by Sirajuddin *et al.* (2007).

We wish to emphasize that our *post mortem* analysis of the work done and the model proposed by Sirajuddin *et al.* (2007) is not intended as any criticism of those investigators. Quite the contrary, progress in science relies upon models based on the best available evidence at the time, and honest, unbiased interpretation thereof; mistakes are an inevitable part of the process. As should be clear from the above commentary, the work carried out by Sirajuddin *et al.* (2007) was welcomed, ground-breaking and greatly stimulated further work in the field. Nonetheless, we felt it was incumbent on us to grope for possible answers to explain the discrepancy between the subunit order deduced then and the revised subunit order demanded by the more recent studies reviewed here. What Sirajuddin *et al.* (2007) got right was the order of SEPT2, SEPT6 and SEPT7 in their hetero-trimeric state; what the new work by Mendonça *et al.* revises is how the trimer composed of those septin classes self-associates to form a hexameric complex. Ultimately, high-resolution structural information from X-ray diffraction of crystals, like that performed by Sirajuddin *et al.* (2007), and/or electron diffraction of cryo-EM images will be required to fully understand, in detail, the molecular contacts responsible for dictating the order of subunit assembly in any given complement of septins.

### Why do the new findings matter?

Why is it important to know the organization of subunits within a septin complex? Septin subunits are not interchangeable, as evidenced by distinct effects of depletion/mutation of individual septins and by distinct expression patterns in different cell types and distinct localization patterns in the same cell types. Also, different septins have distinct molecular features, such as extended N or C termini with specific properties, but also varying abilities to hydrolyze (and maybe even to bind) guanosine triphosphate. Lipids and non-septin proteins interact with specific septins, thus understanding how septin complexes associate with membranes and other proteins requires knowing where each septin resides within a complex. The idea that octamers are made by adding SEPT3-group septins to the ends painted a picture in which pre-existing hexamers could be readily converted to octamers following a simple induction of SEPT3-group septin gene expression. Now that we know SEPT3-group septins are at the center of hetero-octamers, we must ask whether hexamer-to-octamer conversion is even possible, or if, instead, octamer assembly requires new synthesis of all the subunits. Finally, there is growing evidence that individual septins may have functional roles outside of septin hetero-oligomers. How these “lone wolf” septins stay free of other septins is an important question for which appropriate experiments will require an accurate picture of septin organization. Many exciting adventures await those who seek further insights about septins and their biological functions.

### ACKNOWLEDGMENTS:

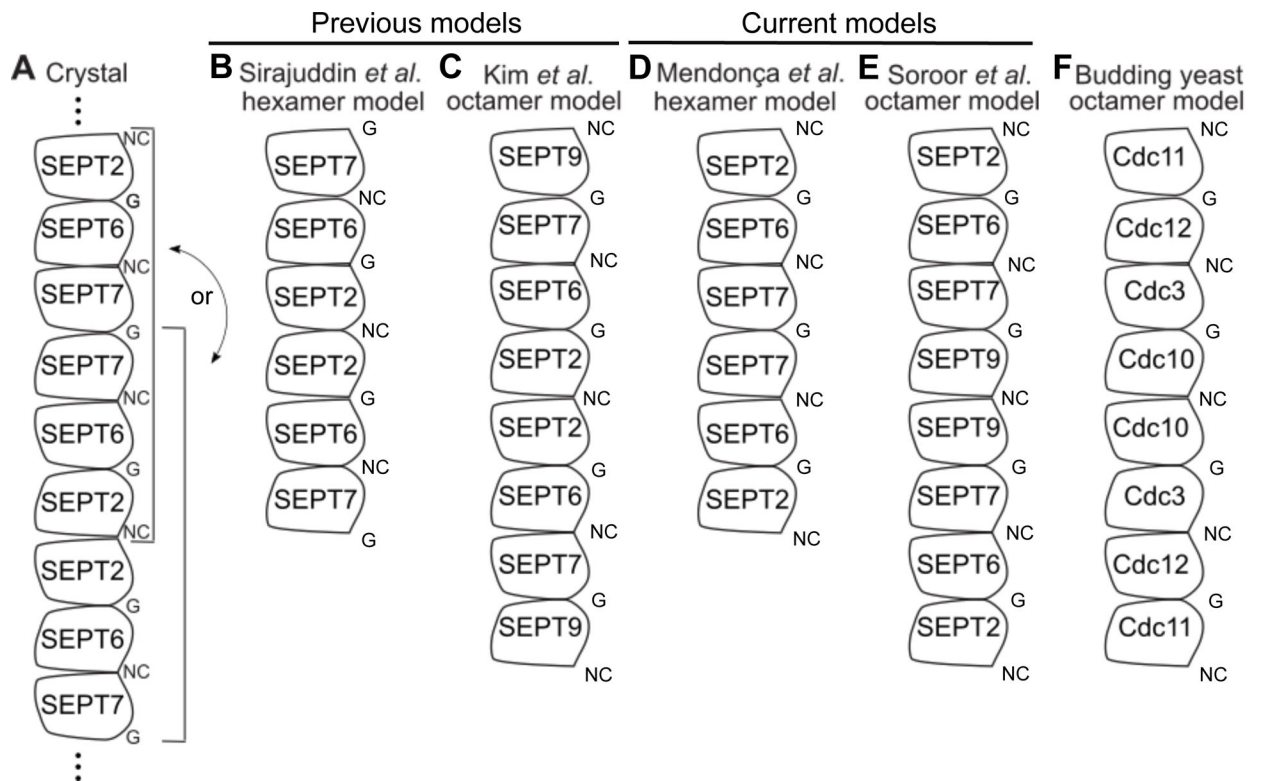
Work on septin assembly in the McMurray lab is supported by NIH R01 Research Grant GM124024 (to M.A.M.). Septin research in the Thorner lab is supported by NIH R01 Research Grants GM101314 and GM21841 (to J.T.). We thank William S. Trimble (Toronto, Canada) for the communication of unpublished results from his laboratory concerning reassessment of the order of subunits in human septin complexes.

## REFERENCES:

- Bertin A, McMurray MA, Grob P, Park S-S, Garcia G, Patanwala I, ... Nogales E (2008). *Saccharomyces cerevisiae* septins: Supramolecular organization of heterooligomers and the mechanism of filament assembly. *Proceedings of the National Academy of Sciences of the United States of America*, 105(24), 8274–8279. 10.1073/pnas.0803330105 [PubMed: 18550837]
- Bertin A, McMurray MA, Thai L, Garcia G, Votin V, Grob P, ... Nogales E (2010). Phosphatidylinositol-4,5-bisphosphate promotes budding yeast septin filament assembly and organization. *Journal of Molecular Biology*, 404(4), 711–731. 10.1016/j.jmb.2010.10.002 [PubMed: 20951708]
- Garcia W, de Araújo APU, Lara F, Foguel D, Tanaka M, Tanaka T, & Garratt RC (2007). An intermediate structure in the thermal unfolding of the GTPase domain of human septin 4 (SEPT4/Bradeion-beta) forms amyloid-like filaments in vitro. *Biochemistry*, 46(39), 11101–11109. 10.1021/bi700702w [PubMed: 17764158]
- Gladfelter AS, & Montagna C (2007). Seeking truth on Monte Verita. Workshop on the molecular biology and biochemistry of septins and septin function. *EMBO Reports*, 8(12), 1120–1126. 10.1038/sj.embor.7401116 [PubMed: 17975554]
- Hernández-Rodríguez Y, Masuo S, Johnson D, Orlando R, Smith A, Couto-Rodriguez M, & Momany M (2014). Distinct Septin Heteropolymers Co-Exist during Multicellular Development in the Filamentous Fungus *Aspergillus nidulans*. *PloS One*, 9(3), e92819. 10.1371/journal.pone.0092819 [PubMed: 24664283]
- Hu H, Yu W, Li S, Ding X, Yu L, & Bi R-C (2006). Crystallization and preliminary crystallographic studies of human septin 1 with site-directed mutations. *Acta Crystallographica. Section F, Structural Biology and Crystallization Communications*, 62(Pt 2), 128–132. 10.1107/S1744309105043228 [PubMed: 16511282]
- John CM, Hite RK, Weirich CS, Fitzgerald DJ, Jawhari H, Faty M, ... Steinmetz MO (2007). The *Caenorhabditis elegans* septin complex is nonpolar. *The EMBO Journal*, 26(14), 3296–3307. 10.1038/sj.emboj.7601775 [PubMed: 17599066]
- Khairat JE, Balasubramaniam V, Othman I, Omar AR, & Hassan SS (2017). Interaction of Recombinant Gallus gallus SEPT5 and Brain Proteins of H5N1-Avian Influenza Virus-Infected Chickens. *Proteomes*, 5(3). 10.3390/proteomes5030023
- Kim MS, Froese CD, Estey MP, & Trimble WS (2011). SEPT9 occupies the terminal positions in septin octamers and mediates polymerization-dependent functions in abscission. *The Journal of Cell Biology*, 195(5), 815–826. 10.1083/jcb.201106131 [PubMed: 22123865]
- Kinoshita M, Field CM, Coughlin ML, Straight AF, & Mitchison TJ (2002). Self- and actin-templated assembly of Mammalian septins. *Developmental Cell*, 3(6), 791–802. [PubMed: 12479805]
- Low C, & Macara IG (2006). Structural analysis of septin 2, 6, and 7 complexes. *The Journal of Biological Chemistry*, 281(41), 30697–30706. 10.1074/jbc.M605179200 [PubMed: 16914550]
- Lukoyanova N, Baldwin SA, & Trinick J (2008). 3D Reconstruction of Mammalian Septin Filaments. *Journal of Molecular Biology*, 376(1), 1–7. 10.1016/j.jmb.2007.11.029 [PubMed: 18083193]
- McMurray MA, Bertin A, Garcia G 3rd, Lam L, Nogales E, & Thorner J (2011). Septin filament formation is essential in budding yeast. *Developmental Cell*, 20(4), 540–549. 10.1016/j.devcel.2011.02.004 [PubMed: 21497764]
- Mendonça DC, Macedo JN, Itri R, Guimaraes SL, Silva F. L. B. da, Cassago A, ... Araujo APU (2019). Repositioning septins within the core particle. *BioRxiv*, 569251. 10.1101/569251
- Mendoza M, Hyman AA, & Glotzer M (2002). GTP binding induces filament assembly of a recombinant septin. *Current Biology: CB*, 12(21), 1858–1863. [PubMed: 12419187]
- Nagata K, Asano T, Nozawa Y, & Inagaki M (2004). Biochemical and cell biological analyses of a mammalian septin complex, Sept7/9b/11. *The Journal of Biological Chemistry*, 279(53), 55895–55904. 10.1074/jbc.M406153200 [PubMed: 15485874]
- Pan F, Malmberg RL, & Momany M (2007). Analysis of septins across kingdoms reveals orthology and new motifs. *BMC Evolutionary Biology*, 7, 103. 10.1186/1471-2148-7-103 [PubMed: 17601340]

- Pissuti Damalio JC, Garcia W, Alves Macêdo JN, de Almeida Marques I, Andreu JM, Giraldo R, ... Ulian Araújo AP (2012). Self assembly of human septin 2 into amyloid filaments. *Biochimie*, 94(3), 628–636. 10.1016/j.biochi.2011.09.014 [PubMed: 21967827]
- Sellin ME, Sandblad L, Stenmark S, & Gullberg M (2011). Deciphering the rules governing assembly order of mammalian septin complexes. *Molecular Biology of the Cell*, 22(17), 3152–3164. 10.1091/mbc.E11-03-0253 [PubMed: 21737677]
- Sellin ME, Stenmark S, & Gullberg M (2014). Cell type-specific expression of SEPT3-homology subgroup members controls the subunit number of heteromeric septin complexes. *Molecular Biology of the Cell*, 25(10), 1594–1607. 10.1091/mbc.E13-09-0553 [PubMed: 24648497]
- Serrão VHB, Alessandro F, Caldas VEA, Marçal RL, Pereira HD, Thiemann OH, & Garratt RC (2011). Promiscuous interactions of human septins: The GTP binding domain of SEPT7 forms filaments within the crystal. *FEBS Letters*, 585(24), 3868–3873. 10.1016/j.febslet.2011.10.043 [PubMed: 22064074]
- Sheffield PJ, Oliver CJ, Kremer BE, Sheng S, Shao Z, & Macara IG (2003). Borg/septin interactions and the assembly of mammalian septin heterodimers, trimers, and filaments. *The Journal of Biological Chemistry*, 278(5), 3483–3488. 10.1074/jbc.M209701200 [PubMed: 12446710]
- Sirajuddin M (2007). Structural studies on mammalian septins—New insights into filament formation (Universität Dortmund Dortmund). Retrieved from [https://pure.mpg.de/pubman/faces/ViewItemOverviewPage.jsp?itemId=item\\_1812789](https://pure.mpg.de/pubman/faces/ViewItemOverviewPage.jsp?itemId=item_1812789)
- Sirajuddin M, Farkasovsky M, Hauer F, Kühlmann D, Macara IG, Weyand M, ... Wittinghofer A (2007). Structural insight into filament formation by mammalian septins. *Nature*, 449(7160), 311–315. 10.1038/nature06052 [PubMed: 17637674]
- Soroor F, Kim MS, Palander O, Balachandran Y, Collins R, Benlekbir S, ... Trimble WS (2019). Revised subunit order of mammalian septin complexes explains their in vitro polymerization properties. *BioRxiv*, 569871 10.1101/569871
- Weirich CS, Erzberger JP, & Barral Y (2008). The septin family of GTPases: Architecture and dynamics. *Nature Reviews. Molecular Cell Biology*, 9(6), 478–489. 10.1038/nrm2407 [PubMed: 18478031]
- Wiesemann K, Simm S, Mirus O, Ladig R, & Schleiff E (2019). Regulation of two GTPases Toc159 and Toc34 in the translocon of the outer envelope of chloroplasts. *Biochimica Et Biophysica Acta. Proteins and Proteomics*, 1867(6), 627–636. 10.1016/j.bbapap.2019.01.002 [PubMed: 30611779]
- Zent E, Vetter I, & Wittinghofer A (2011). Structural and biochemical properties of Sept7, a unique septin required for filament formation. *Biological Chemistry*, 392(8–9), 791–797. 10.1515/BC.2011.082 [PubMed: 21824007]



**Figure 1.**

Past and current models of the subunit arrangement within mammalian septin heterooligomers. (A) As described in Sirajuddin *et al.* (2007), within the crystals formed by a purified hexameric complex of human SEPT2, SEPT6, and SEPT7, the asymmetric unit was a SEPT2-SEPT6-SEPT7 hetero-trimer, but because the septins polymerized within the crystals into continuous filaments, it was not immediately obvious how the hexamer was organized within the filaments. The alternating “G” and “NC” interfaces were defined and characterized in that study. (B) Based on their interpretation of EM images of MBP-SEPT2-labeled hexamers recovered from solution, Sirajuddin *et al.* (2007) proposed a model in which SEPT2 forms a central homodimer via the NC interface. (C) Influenced by the model in (B), Kim *et al.* (2011) proposed a model in which a fourth human septin, SEPT9, interacts with SEPT2, SEPT6, and SEPT7 to form hetero-octamers in which two SEPT9 molecules occupy positions at the ends of the octamers. (D) New work by Mendonça *et al.* in this issue provides clear evidence of a distinctly different hexameric organization, in which SEPT7 forms the central homodimer. (E) A revised model for human octamer organization based on recent work by Soroor, *et al.* (2019); see text for further details. In this new model, the organization of a mammalian septin hetero-octamer is congruent with that determined for a yeast hetero-octamer. (F) In septin octamers found in budding yeast cells, Cdc10, the closest yeast relative of SEPT9, occupies a central homodimer, and octamers polymerize into filaments via a salt-sensitive Cdc11 NC homodimer, as determined by Bertin *et al.* (2008).