Title

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

Journal

Cytoskeleton (Hoboken, N.J.), 76(9-10)

ISSN

1949-3584

Authors

McMurray, Michael A

Thorner, Jeremy

Publication Date

2019-09-01

DOI

10.1002/cm.21571

Peer reviewed

Received: 24 September 2019 | Revised: 2 October 2019 | Accepted: 8 October 2019

DOI: 10.1002/cm.21571

WILEY

COMMENTARY

Turning it inside out: The organization of human septin heterooligomers

Michael A. McMurray[1] | Jeremy Thorner[2,3,4]

[1]Department of Cell and Developmental Biology, University of Colorado Anschutz Medical Campus, Aurora, Colorado

[2]Division of Biochemistry, Biophysics & Structural Biology, University of California, Berkeley, California

[3]Division of Cell & Developmental Biology, University of California, Berkeley, California

[4]Department of Molecular and Cell Biology, University of California, Berkeley, California

Correspondence
Jeremy Thorner, Department of Molecular and Cell Biology, University of California, Berkeley, CA 94720.
Email: jthorner@berkeley.edu

Funding information
NIH, Grant/Award Numbers: GM21841, GM101314, GM124024

Abstract

Septin family proteins are quite similar to each other both within and between eukaryotic species. Typically, multiple discrete septins co-assemble into linear heterooligomers (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, that is, members of the same group appear to occupy the same position within oligomeric septin protomers, which are "palindromic" (have twofold 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 heterohexamers 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 heterooctamers, 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 heterohexamers and heterooctamers is inverted relative to the original model. These findings are discussed here in a broader context, including possible causes for the initial confusion.

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

1 | 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 the translocation of precursor proteins into chloroplasts) forms a homodimer (Wiesemann, Simm, Mirus, Ladig, & Schleiff, 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

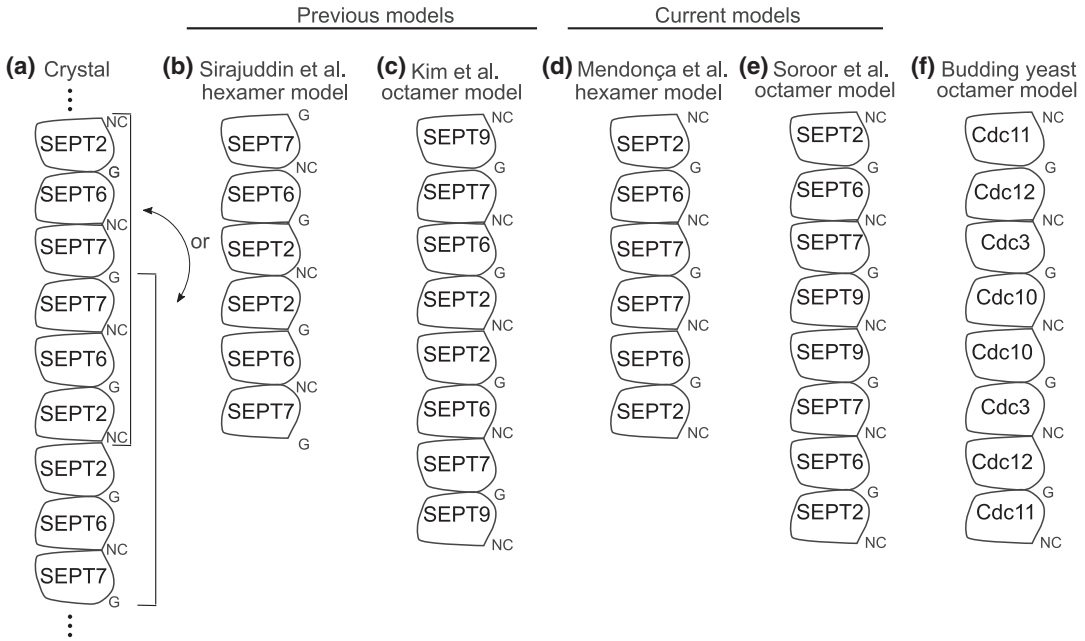

FIGURE 1 Past and current models of the subunit arrangement within mammalian septin heterooligomers. (a) As described in Sirajuddin et al. (2007), within the crystals formed by a purified hexameric complex of human SEPT2, SEPT6, and SEPT7, the asymmetric unit was a SEPT2–SEPT6–SEPT7 heterotrimer, but 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, Froese, Estey, and Trimble (2011) proposed a model in which a fourth human septin, SEPT9, interacts with SEPT2, SEPT6, and SEPT7 to form heterooctamers in which two SEPT9 molecules occupy the 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 heterooctamer is congruent with that determined for a yeast heterooctamer. (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)

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; Pissuti Damalio et al., 2012; Serrão et al., 2011; Zent, Vetter, & Wittinghofer, 2011). Given these properties, how do such similar proteins heterooligomerize, and how is each subunit directed to occupy a defined position within the heterooligomeric 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 the 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, Field, Coughlin, Straight, & Mitchison, 2002; Nagata, Asano, Nozawa, & Inagaki, 2004). From the 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. (2007) determined that the complex was a linear heterohexamer and proposed a model for the position of each of the three human septins in the heterohexamer (Figure 1).

2 | CHALLENGES IN DETERMINING THE SUBUNIT ARRANGEMENT IN SEPTIN HETEROOLIGOMERS

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 pairwise or in even greater combinations, interactions among them can be readily detected in vitro by standard biochemical methods (such as co-immunoprecipitation, size exclusion chromatography, glycerol gradient sedimentation). 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 a purified form. However, instability, misfolding, and aggregation of individually expressed full-length septins have often been a problem (Garcia et al., 2007; Hu et al., 2006; Khairat, Balasubramaniam, Othman, Omar, & Hassan, 2017; Pissuti Damalio et al., 2012), 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 heterodimerizes 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 et al., 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 heterooligomeric 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 copurification of a complex containing a 1:1:1 ratio of each septin component 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 copurified as heterodimers in every case (Sheffield et al., 2003), a result incompatible with a linear subunit arrangement in the heterohexameric 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 where an individual subunit resides within a septin heterooligomer by using EM. First, if an antibody uniquely specific for the septin of interest is available (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 heterooctameric septin complex from budding yeast (Bertin et al., 2008). However, this approach was not used by Sirajuddin et al. (2007) 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 elected 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. (2007) 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 native SEPT6 (434 residues) and native SEPT7 (437 residues) in bacterial cells from which they purified the resulting complexes. 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(Δ316–361) 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(Δ316–361), 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 heterohexamer.

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 nonspecific 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, 2007]. 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 green fluorescent protein (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 is was fused (John et al., 2007). In the work by Sirajuddin et al. (2007), only a single image of just one unaveraged hexamer was provided in 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. (2007) used this approach to solve the crystal structure of their SEPT2-, SEPT6- and SEPT7-containing complexes to about 4Å resolution. 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. (2007) 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 heterohexamer 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 heterohexamer is inverted from that proffered by Sirajuddin et al., namely SEPT2–SEPT6–SEPT7–SEPT7–SEPT6–SEPT2 (see Figure 1d).

3 | 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 and 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 that Sirajuddin et al. observed by using 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 Figure 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 Figure 3c in Sirajuddin et al., 2007]. By contrast, no kinks were observed in yeast septin rods examined under the same conditions [see Figure 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 to the fact that the hexameric particles examined were composed solely of the rat septins Sept3, Sept5, and Sept7 (Lukoyanova et al., 2008) remains questionable and would represent an arrangement incompatible with contemporary understanding of mammalian septin heterooligomer 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

heterooctamers (Figure 1c), and others reached the same conclusion for similar reasons for human SEPT9 and other SEPT3 group members (Sellin, Sandblad, Stenmark, & Gullberg, 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 et al., 2007), and it was demonstrated convincingly that a Cdc10-Cdc10 NC homodimer resides at the *center* of the yeast septin heterooctamers, 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?

4 | EVIDENCE DEMANDING A REVISED ORDER OF SUBUNITS IN HUMAN SEPTIN HETEROOLIGOMERS

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 heterohexamers as proposed by Sirajuddin et al. (2007) and within heterooctamers 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 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 heterohexamers 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 heterooctamers.

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 et al., 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 clear 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 heterohexamers.

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 heterohexamers containing MBP–SEPT2, SEPT6, and SEPT7, and, in their hands of these investigators, the hexameric rods were unkinked 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 1(c)). 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 copolymerize in low salt, because they differ in which septin–septin interface is exposed at their termini (Figure 1b,c). In contrast, purified hexamers and octamers readily copolymerized in vitro. These results strongly support an order of subunits in the human heterooctamers of SEPT2–SEPT6–SEPT7–SEPT9–SEPT9–SEPT7–SEPT6–SEPT2 (Figure 1e). Thus, the overall organization of the human heterooctamer now is essentially congruent with that determined for yeast septin heterooctamers (Bertin et al., 2008) (Figure 1e,f).

5 | 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. (2019) 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". 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 prefixation 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 truncated septin behaved as a mix of monomers and homodimers; within the asymmetric unit of the crystal, the truncated SEPT2 homodimerized via its both 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. (2007) were able to determine that, in solution, SEPT2 homodimers formed preferentially via their G interface. 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 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 the disassembly of yeast septin filaments into its constituent heterooctameric 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., 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 heterooligomers, 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 analysis tool to predict the salt sensitivity of the SEPT2 NC homodimer interface compared with that of the SEPT7 G homodimer, and it has been 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 heterooctamers compared with 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 postmortem 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, groundbreaking, 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 heterotrimeric 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.

6 | 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 the 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 heterooctamers, 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 heterooligomers. 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 and Minhajuddin Sirajuddin (Bangalore, India), Alfred Wittinghofer (Dortmund, Germany), Roberto Dominguez (Baltimore, MD), and an anonymous reviewer for their careful reading of the manuscript and their helpful suggestions.

ORCID

Jeremy Thorner https://orcid.org/0000-0002-2583-500X

REFERENCES

Bertin, A., McMurray, M. A., 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. https://doi.org/10.1073/pnas.0803330105

Bertin, A., McMurray, M. A., 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. https://doi.org/10.1016/j.jmb.2010.10.002

Garcia, W., de Araújo, A. P. U., Lara, F., Foguel, D., Tanaka, M., Tanaka, T., & Garratt, R. C. (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. https://doi.org/10.1021/bi700702w

Gladfelter, A. S., & 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. https://doi.org/10.1038/sj.embor.7401116

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. https://doi.org/10.1371/journal.pone.0092819

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*(2), 128–132. https://doi.org/10.1107/S1744309105043228

John, C. M., Hite, R. K., Weirich, C. S., Fitzgerald, D. J., Jawhari, H., Faty, M., … Steinmetz, M. O. (2007). The *Caenorhabditis elegans* septin complex is nonpolar. *The EMBO Journal*, *26*(14), 3296–3307. https://doi.org/10.1038/sj.emboj.7601775

Khairat, J. E., Balasubramaniam, V., Othman, I., Omar, A. R., & Hassan, S. S. (2017). Interaction of recombinant *Gallus gallus* SEPT5 and brain proteins of H5N1-avian influenza virus-infected chickens. *Proteomes*, *5*(3), e23.1–e23.17. https://doi.org/10.3390/proteomes5030023

Kim, M. S., Froese, C. D., Estey, M. P., & Trimble, W. S. (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. https://doi.org/10.1083/jcb.201106131

Kinoshita, M., Field, C. M., Coughlin, M. L., Straight, A. F., & Mitchison, T. J. (2002). Self- and actin-templated assembly of mammalian septins. *Developmental Cell*, *3*(6), 791–802.

Low, C., & Macara, I. G. (2006). Structural analysis of septin 2, 6, and 7 complexes. *The Journal of Biological Chemistry*, *281*(41), 30697–30706. https://doi.org/10.1074/jbc.M605179200

Lukoyanova, N., Baldwin, S. A., & Trinick, J. (2008). 3D reconstruction of mammalian septin filaments. *Journal of Molecular Biology*, *376*(1), 1–7. https://doi.org/10.1016/j.jmb.2007.11.029

McMurray, M. A., 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. https://doi.org/10.1016/j.devcel.2011.02.004

Mendonça, D. C., Macedo, J. N., Itri, R., Guimaraes, S. L., da Silva, F. L. B., Cassago, A., … Araujo, A. P. U. (2019). Repositioning septins within the core particle. *bioRxiv*, *569251*, 1–20. https://doi.org/10.1101/569251

Mendoza, M., Hyman, A. A., & Glotzer, M. (2002). GTP binding induces filament assembly of a recombinant septin. *Current Biology: CB*, *12*(21), 1858–1863.

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. https://doi.org/10.1074/jbc.M406153200

Pan, F., Malmberg, R. L., & Momany, M. (2007). Analysis of septins across kingdoms reveals orthology and new motifs. *BMC Evolutionary Biology*, *7*, 103. https://doi.org/10.1186/1471-2148-7-103

Pissuti Damalio, J. C., Garcia, W., Alves Macêdo, J. N., de Almeida Marques, I., Andreu, J. M., Giraldo, R., … Ulian Araújo, A. P. (2012). Self assembly of human septin 2 into amyloid filaments. *Biochimie*, *94*(3), 628–636. https://doi.org/10.1016/j.biochi.2011.09.014

Sellin, M. E., 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. https://doi.org/10.1091/mbc.E11-03-0253

Sellin, M. E., 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. https://doi.org/10.1091/mbc.E13-09-0553

Serrão, V. H. B., Alessandro, F., Caldas, V. E. A., Marçal, R. L., Pereira, H. D., Thiemann, O. H., & Garratt, R. C. (2011). Promiscuous interactions of human septins: The GTP binding domain of SEPT7 forms filaments within the crystal. *FEBS Letters*, *585*(24), 3868–3873. https://doi.org/10.1016/j.febslet.2011.10.043

Sheffield, P. J., Oliver, C. J., Kremer, B. E., Sheng, S., Shao, Z., & Macara, I. G. (2003). Borg/septin interactions and the assembly of mammalian septin heterodimers, trimers, and filaments. *The Journal of Biological Chemistry*, *278*(5), 3483–3488. https://doi.org/10.1074/jbc.M209701200

Sirajuddin, M. (2007). Structural studies on mammalian septins—New insights into filament formation, Ph.D. Thesis, Universität Dortmund, Dortmund. Retrieved from https://pure.mpg.de/pubman/faces/ViewItemOverviewPage.jsp?itemId=item_1812789.

Sirajuddin, M., Farkasovsky, M., Hauer, F., Kühlmann, D., Macara, I. G., Weyand, M., … Wittinghofer, A. (2007). Structural insight into filament formation by mammalian septins. *Nature*, *449*(7160), 311–315. https://doi.org/10.1038/nature06052

Soroor, F., Kim, M. S., Palander, O., Balachandran, Y., Collins, R., Benlekbir, S., … Trimble, W. S. (2019). Revised subunit order of mammalian septin complexes explains their in vitro polymerization properties. *BioRxiv*. 1–34. https://doi.org/10.1101/569871

Weirich, C. S., Erzberger, J. P., & Barral, Y. (2008). The septin family of GTPases: Architecture and dynamics. *Nature Reviews. Molecular Cell Biology*, *9*(6), 478–489. https://doi.org/10.1038/nrm2407

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. https://doi.org/10.1016/j.bbapap.2019.01.002

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. https://doi.org/10.1515/BC.2011.082

How to cite this article: McMurray MA, Thorner J. Turning it inside out: The organization of human septin heterooligomers. *Cytoskeleton*. 2019;76:449–456. https://doi.org/10.1002/cm.21571