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Assembly of Mammalian Septins

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Septins are a conserved family of polymerizing guanine nucleotide binding proteins associated with diverse processes in dividing and non-dividing cells. In humans, 12 septin genes generate dozens of polypeptides, many of which comprise heterooligomeric complexes. Native and recombinant mammalian septin complexes are purified as ~8-nm-thick filaments of variable length. Ultrastructurally, a mammalian septin filament appears an irregular array of structural segments, whose polarity is obscure. The filaments have a potential to self-assemble into higher-order structures by lateral stacking and tandem annealing, eventually forming uniformly curved bundles, *i.e.*, rings and coils. The septin filaments also undergo templated assembly along existing actin bundles containing an adapter protein, anillin. The resultant higher-order assembly of septin filaments may provide scaffolds to recruit other molecules and/or help organize the actin-based structures. The *in vitro* self-assembly is an irreversible process, which is not coupled with robust nucleotide exchange or hydrolysis. In contrast, septin-based structures rearrange and disassemble in cells, which might be controlled by diverse factors (*e.g.*, the Cdc42-borg system, anillin, syntaxin, phospholipids) and covalent modifications (*e.g.*, phosphorylation, ubiquitination, sumoylation). An immediate goal of septin biochemistry is to define the mechanisms of assembly and disassembly of this elusive cytoskeleton.

Key words: cytokinesis, exocytosis, GTPase, self-assembly.

The septin assembly was originally discovered by electron microscopy as "mother-bud neck filaments" in budding yeast (*1*). Electron-dense, ~10-nm-thick striations run circumferentially between a mother cell and the bud, and these were later identified with the "septin rings" observed by fluorescence microscopy. Since septin mutants are commonly defective in cytokinesis and formation of the neck filaments/septin rings, septins have been considered to be the primary constituents of the neck filaments (*2*, *3*). To date, genetic and cytological studies have revealed that the septin rings may serve as (i) a spatial landmark to establish and/or maintain cell polarity for budding, (ii) a diffusion barrier to segregate cortical molecules between mother and bud, and (iii) a scaffold for anchoring other proteins or higher-order subcellular structures (*2–9*). Some of these functions may be unique to yeast mitosis, but requirement for septins in cytokinesis is likely to hold true to metazoan systems (*10–12*). Biochemical and ultrastructural analyses of septin complexes purified from *Drosophila* embryo provided evidence of their (i) enzymatic activities for GTP-exchange and -hydrolysis, (ii) filamentous ultrastructure, and 3) tendency toward self-assembly (*13*). Assembly and disassembly of the septin subunits or filaments have been speculated to be coupled with their enzymatic activities, although the details are as yet unknown. Recent development of the recombinant septin complexes has opened up an avenue to address long-standing questions about these mysterious proteins in higher eukaryotes.

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1. Complexity of the mammalian septin system

The prototype of the septin complex was isolated from *Drosophila* embryo by immunoaffinity chromatography for one of its subunits, Pnut (*13*). The ~8-nm-thick filamentous complexes are composed of three septin polypeptides, Sep1, Sep2 and Pnut, with a stoichiometry of ~1:1:1. Mammalian septin complexes purified from tissues and cells are ultrastructurally similar to the fly septin complexes (*14*, *15*). However, each preparation contains far more than three proteins with an obscure stoichiometry, suggestive of mixed complexes of distinct composition. This is due to a higher complexity and redundancy of the mammalian septin system (*i.e.*, 5 septin genes in *Drosophila vs.* 12 in humans and mice).

A simple mammalian septin complex with a ~1:1:1 stoichiometry was fortuitously isolated from NIH3T3 cell lysate by affinity chromatography with GST-fused borg3, a Cdc42-interacting protein (*16*). The three components, Sept2, Sept6 and Sept7, are orthologs of the *Drosophila* Sep1, Sep2 and Pnut, respectively. Thus, the simple mammalian septin complex is regarded as a counterpart of the original fly septin complex.

2. Dissecting the complexity of the septin system by reconstitution

The Sept2/6/7 complex serves as a model for biochemical and structural analyses of the mammalian septin complex. However, attempts to reconstitute the complex *in vitro* from individual recombinant polypeptides have been unsuccessful. This is mainly because (i) Sept7 is virtually insoluble by itself either in bacteria (*16*, *17*) or in eukaryotic cells (*15*), and (ii) Sept2 and Sept6 can form homo- and hetero-oligomers (*15–17*, *18*). In contrast, bi-

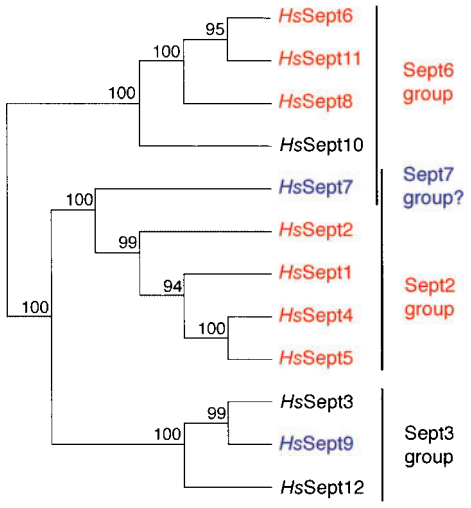

Fig. 1. **A phylogenetic tree of the human septins.** The longest amino acid sequence among the putative polypeptides generated by each gene was analyzed with the software CLUSTALW (Method: UPGMA with bootstrap of 1000 and systematic tie-breaking. Distance: Poisson-corrected with proportionally distributed gaps.). Septins with acidic, neutral and basic isoelectric points are respectively colored red, black and blue. Refer to Ref. *40* for the mammalian septins nomenclature.

cistronic co-expression of Sept6 and Sept7 yields a soluble heterodimeric complex of Sept6/7 with a stoichiometry of ~1:1 (*15*–*17*). It is speculated that the heterodimer formation, which requires carboxyl-terminal coiled-coil regions of Sept6 and Sept7 (*17*), is critical for their correct folding and/or preventing the aggregation of Sept7. Although incomplete septin complexes and free septin polypeptides may be subject to degradation in fly and mouse cells (*19*, *20*, *15*), partial septin complexes lacking one subunit are stable in yeast (*21*). Taken together, these findings indicate that septin preparations may not only consist of the canonical complexes, but potentially contain traces of hetero-oligomeric partial complexes, homo-oligomers and free polypeptides.

By co-expression of Sept2 with the Sept6/7 partial complex, stable Sept2/6/7 complexes with ~1:1:1 stoichiometry have become available. Bacterially expressed Sept2/6/7 complexes are mostly particles with a size close to 256 kilodalton, the molecular mass of the putative 2:2:2 hexamer (*17*). In contrast, when Sept2/6/7 polypeptides are co-expressed in insect cells, filamentous complexes with larger molecular size predominate (*15*). The elution profiles of gel filtration chromatography have no peak around the hexamer size, as is the case with the native mammalian complexes. Thus, the bacterial environment might not be favorable for formation or maintenance of septin filaments. It is well known that generation of functional actin monomers and tubulin heterodimers depends on specific chaperone systems. Perhaps the folding of septin polypeptides and formation of functional oligomers might likewise require some active mechanism.

Unlike the fly and yeast septin filaments, clear structural periodicity of the native mammalian septin filaments has not been consistently demonstrated by electron microscopy (*14*, *15*). Such an irregularity may be attributable, at least in part, to mixed complexes of various composition. However, the recombinant Sept2/6/7 filaments expressed in either eukaryotic or bacterial system also lack structural periodicity that indicates the unit hexamer (*15*, *17*). The fly and yeast septin filaments, which are composed mostly of canonical unit complexes, also contain filaments with irregular lengths (*13*, *21*). Thus, some irregularity might be inherent in the septin filament architecture. Further careful studies should elucidate the structure and polarity issues of the septin filaments.

3. Diversity and redundancy of the mammalian septin system

The mammalian septin family members can be classified into four groups by amino acid sequence homology (Figs. 1 and 2) (*22*). The homology-based classification so far agrees with their compatibility in the recombinant complex formation tested in insect cells (*18*). (i) Sept1, Sept2, Sept4 and Sept5 can each form an equimolar complex with the Sept6/7 partial complex, giving rise to SeptX/6/7 complexes of ~1:1:1 stoichiometry (X = 1, 2, 4 or 5). (ii) Sept6 in the Sept2/6/7 complex is replaceable with Sept11, and probably with Sept8 or Sept10. (iii) Sept7 is unique and may not be replaceable. (iv) Sept3, Sept9 and Sept12 are predicted to lack a coiled-coil region, unlike the other septins. Exploration for the rules by which they constitute complexes is under way.

Each septin polypeptide seems to have temporally and spatially distinct expression patterns *in vivo* (*15*, *18*, *23*, *25*, *30*). The redundancy and interchangeability among septin subunits may contribute to diversify the repertoire of the septin complexes in a combinatorial fashion. Whether the complexes with distinct composition have distinct biochemical and functional properties remains to be tested. Intriguingly, loss of Sept5 affects exocytosis in mouse platelets (*24*), whereas it is tolerated in the brain probably owing to compensatory up-regulation of several other septins (*20*). Thus, the redundancy and flexibility obviously contribute to secure the mammalian septin systems, while hampering genetic approaches.

4. Self-assembly of the septin filaments

The recombinant Sept2/6/7 complexes expressed in insect cells are purified as filaments in buffers of moderate to high ionic strength (0.1–0.5 M KCl plus 0.1 M imidazole) at a concentration of ~0.1 mg/ml (~0.35 μM as unit hexamer) (*15*). On reducing the ionic strength below 0.15 M KCl by dialysis, the septin filaments elongate by end-to-end joining or annealing (*15*), as do the native septin complexes purified from yeast and fly (*13*, *21*). In addition, the fly and mammalian septin filaments form bundles by lateral stacking, which is not apparent in the yeast septin filaments. As the polarity of each filament is still unclear, it is also unknown whether the annealing and bundling occur in a polar or a random fashion. The recombinant mammalian Sept2/6/7 bundles often appear as curled ribbons. After longer incubation, bundles with uniform curvature, *i.e.*, rings and coils of 0.6±0.1 μm in diameter, predominate. Since these structures are stable after dilution or under high ionic strength, the higher-order assembly of septin filaments seems to be an irreversible process *in vitro*. The assembly occurs in the presence or absence of exogenous nucleotides and the bound

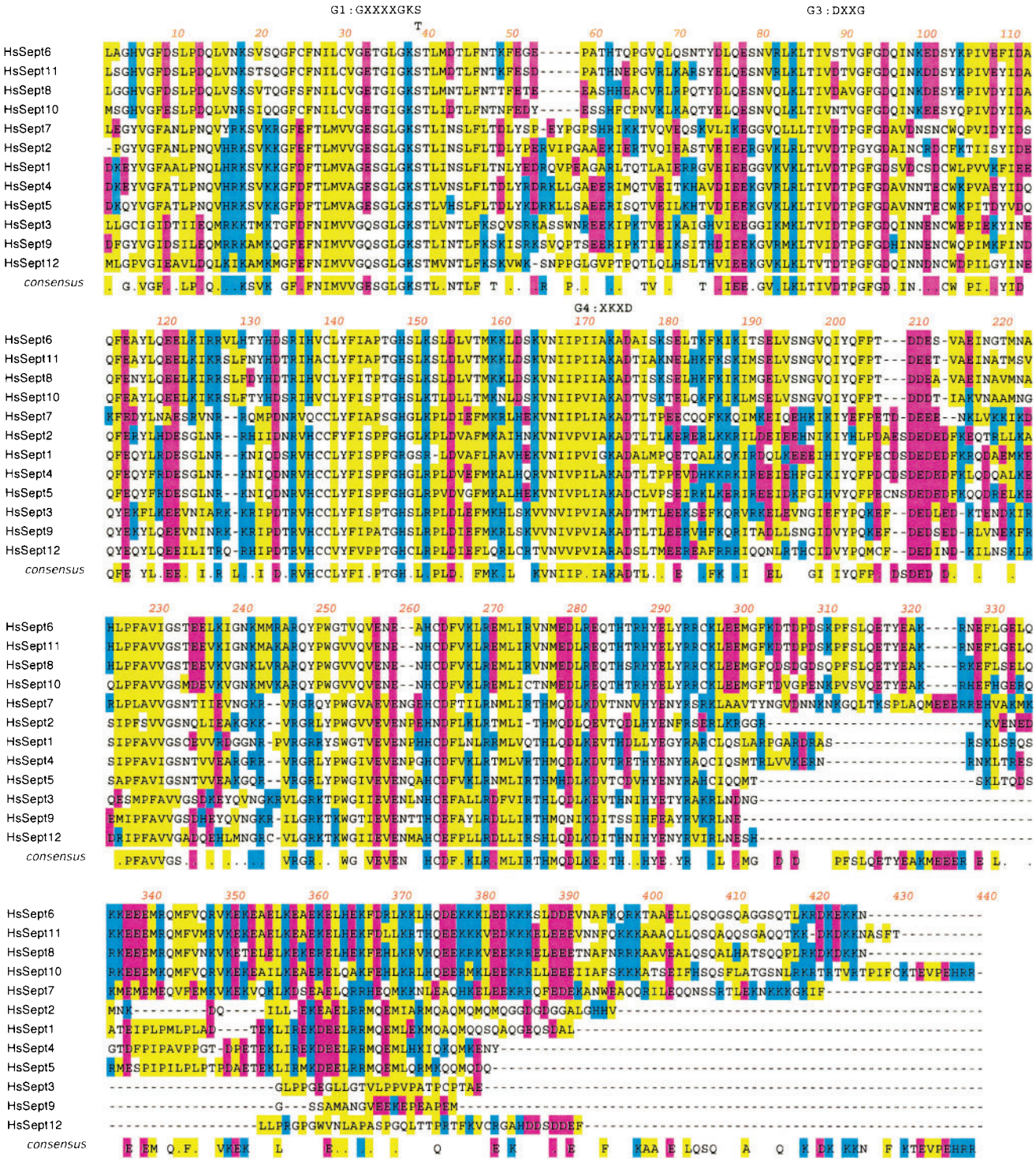

Fig. 2. **Multiple alignment of the human septins removing the amino-terminal regions.** The acidic, basic and hydrophobic residues are respectively colored red, blue and yellow. Note the chimeric structure of Sept7, which is similar to the Sept2 group except for the Sept6-like carboxyl-terminal region starting from around the position 330 (red scale at the top).

GTP/GDP ratio remains unchanged throughout the process (*15*). Thus, the assembly of septin filaments into curved bundles is unlikely to be coupled with the nucleotide exchange or hydrolysis (see section 8).

The physiological relevance of the septin rings *in vitro* has not been established. However, they look almost identical to the septin-based rings that appear in tissue culture cells after actin perturbation (*15*, *25*, *26*). The *in vitro* and cellular septin rings are currently regarded as the default or storage mode of septin assembly free from the actin template (see section 6). As the molecular basis of the higher-order self-assembly is a critical issue of the

septin cytoskeleton, further structural, biochemical and biophysical approaches are necessary to reveal the mechanism underlying their unique behaviors.

5. Septin-based ring structures *in vivo*

Physiological structures reminiscent of the septin rings appear during *Drosophila* spermatogenesis, when cytokinesis arrests after forming contractile rings (*27*). The residual contractile rings eventually lose the actin and contractility, while retaining their annular structures. The resultant intercellular openings of 1–2 μm in diameter, called ring canals, help distribute cytoplasm and organelles among the primordial germ cells. After the dissipation of actin, the major structural proteins in the ring canals are septins and anillin (*27*, *28*; see section 6). Similar septin/anillin-based structures have not been found in mammals, however, structures with comparable size and curvature can be reconstituted *in vitro* with the recombinant mammalian septin filaments and anillin (*18*). These data raises two hypotheses to be tested: (i) mammalian septins plus anillin are sufficient to form and maintain structures similar to *Drosophila* ring canals, and (ii) one of the unique functions of the septin cytoskeletal system is to form and retain rigid concavities of the cell cortex.

6. Actin-templated assembly of the septin filaments

The septin assembly occurs in cultured mammalian cells as accessory bundles tightly associated with the actin-based structures: *i.e.*, contractile ring, cortical actin, and stress fibers (*15*, *16*). Since the Sept2/6/7 filaments do not directly interact with pure actin filaments in a physiological buffer (*15*), the actin-associated septin assembly requires adapter molecules that mediate the indirect interaction between the two filament systems. Anillin, an actin-bundling protein that localizes to the contractile ring during cytokinesis (*26–28*), has been considered as one such adapter. Cytological analysis revealed that anillin's pleckstrin-homology (PH) domain plus a flanking region is necessary to recruit the endogenous septins into ectopic aggregates (*26*). Pure actin filaments bundled by anillin can recruit septin filaments in co-sedimentation assay, which is more obviously demonstrated by a visual assay using fluorescence microscopy (*15*). As predicted, anillin fragments without the PH domain cannot recruit septins onto the actin bundles. The visual assay also showed that septin recruitment does not alter the gross structure of the anillin/actin bundles. In contrast, three other actin-bundling proteins present in contractile ring (α-actinin, filamin, fascin) have no such activity as anillin. Thus, anillin is considered as the principal adapter protein for septins in the contractile ring. On the other hand, another protein is supposed to anchor septin bundles along actin stress fibers in interphase, when anillin is mostly sequestered in the nucleus (*26*, *28*). The identity of this hypothetical adapter protein still remains an open question.

7. Possible other templated assemblies of the septin filaments

The templated assembly mechanism may also explain other subcellular localizations of septins. For instance, septin localization and assembly beneath the plasma membrane in interphase might be due to direct interaction between septins and lipid bilayers, because GST-fused Sept5 can interact with certain phospholipids *in vitro* (*29*). Alternatively, cortical recruitment of septins may be due to indirect mechanisms mediated by membrane-associated proteins known to interact with septins, such as extra-nuclear anillin remaining at the cell cortex in interphase (*18*, *26*) and syntaxins (*24*, *30*).

Besides the typical actin-associated localization, Sept9 is present along microtubules in some cell lines (*31*, *32*). The relationship between microtubules and septins has been little understood, but they may directly interact, as recombinant Sept9 and purified microtubules can co-sediment *in vitro* (*32*). The possible interaction between the two guanine nucleotide-binding cytoskeletal systems is another important subject to be explored in various organisms (*9*).

8. Septins' GTPase activity and assembly

Septins belong to the GTPase superfamily for their conserved GTPase motifs and enzymatic activities. The primary structures are similar to those of the Ras superfamily proteins rather than those of tubulins or FtsZ. The role of septins' GTPase activities has been one of the most challenging problems in septin biochemistry (*2*, *3*). The original *Drosophila* Sep1/Sep2/Pnut complex and the recombinant mammalian Sept2/6/7 complex are purified with tightly bound guanine nucleotides (*13*, *15*). The stoichiometry is one nucleotide per polypeptide, with a GDP:GTP ratio of 2:1–3:1. In the case of the Sept2/6/7 filaments, the GDP:GTP ratio does not apparently change after incubation at 37°C for up to 2 h, indicating that the existing GTP is not hydrolyzed (*18*). In contrast, radiolabeled GTP newly bound to the Sep1/Sep2/Pnut and the Sept2/6/7 complexes is hydrolyzed under similar conditions (*13*, *17*). However, hydrolysis is not robust in either case, probably limited by the preceding sluggish exchange reaction. There is no evidence to support a hypothesis that the higher-order assembly of the Sept2/6/7 filaments depends on exchange and/or hydrolysis of guanine nucleotides as mentioned above (18; section 4). Thus, septins' GTP-hydrolysis might be coupled with disassembly of the higher-order structures, or the process before forming the filamentous complexes.

A recent study using bacterially expressed septins has demonstrated that a part of GTP-hydrolysis occurs upon forming the partial complexes (*17*): during *in vitro* Sept6/7 heterodimer formation, less radioactivity is retained when [γ-^{32}P]GTP is used as the substrate than [α-^{32}P]GTP. These data suggest that hydrolysis and release of a part of the γ-phosphate accompanies the formation of Sept6/7 heterodimer. This might be analogous to the formation of α/β-tubulin heterodimer, in which only α-tubulin hydrolyzes GTP and never exchanges the GDP. As to Sept6/7, the major open questions would be 1) which septin subunit (Sept6, Sept7 or both) hydrolyzes GTP, 2) whether the resultant GDP is exchangeable for GTP, and 3) whether the hydrolysis is necessary for the heterodimer formation.

9. Regulation of septin assembly by covalent modification

The pure septin rings *in vitro* are so stable that they do not self-disassemble even under high ionic strength (*15*).

Thus, additional factors seem to be required for the disassembly of higher-order septin structures. The first clues to this problem have come again from yeast genetics. Yeast Siz1p and Siz2p are E3-like factors required for covalent attachment of the ubiquitin-like protein SUMO to target proteins including septins (*33*). Mutations of the conserved sumoylation sites in septins result in septin rings aberrantly persistent beyond the cell cycle (*34*). Thus, sumoylation of septins controls disassembly and/or clearance of the septin ring, although it is as yet unknown how septin rings disassemble after sumoylation. Some mammalian septins have possible sumoylation sites, and it remains to be tested whether sumoylation, if any, regulates disassembly and/or clearance of the mammalian septin-based structures.

An interesting correlation between phosphorylation status of a yeast septin subunit and dynamics of the septin ring has recently been reported (*35*). Although phosphorylation of septins was first demonstrated in the rat brain, biological significance of Sept3 phosphorylation by cGMP-dependent protein kinase (*36*) is still unknown. Further studies should reveal whether phosphorylation and dephosphorylation play a critical role in spatial and temporal regulation of the septin assembly and functions in various situations.

10. Aberrant deposit of septins in neurodegeneration

In addition to the physiological assembly, a few septins have been found to accumulate into pathological cytoplasmic structures in common neurodegenerative disorders in humans: *e.g.*, neurofibrillary tangles and glial tangles in Alzheimer's disease (major component: a microtubule-associated protein, tau) (*37*), and Lewy bodies and glial cytoplasmic inclusions in Parkinson's disease and other synucleinopathies (major component: α-synuclein) (*38*). In either case, the septins may not deposit as the canonical complexes, because Sept7 has never been detected in the aggregates. A plausible interpretation would be direct or indirect adsorption and/or entanglement of septins onto existing tau-based or α-synuclein-based polymers. However, some septins may facilitate formation of the core structures, because α-synuclein and Sept4 expressed in tissue culture cells co-sediment and co-aggregate in a reciprocal fashion (*38*). The biochemical and pathological relevance of these results should be rigorously tested *in vitro* and *in vivo*.

Sept5 has been re-discovered as one of the substrates of an E3 ubiquitin protein ligase, parkin, whose loss-of-function mutations cause juvenile parkinsonism (*39*). Possible accumulation of Sept5 and its structural homolog Sept4 is suspected to play a role in the pathogenesis of Parkinson's disease. Further information on the degradation pathways of septins and the septin status in the aberrant structures (*e.g.*, size, structure, stoichiometry, nucleotide content, modifications) should serve as a clue to reveal the pathophysiology underlying these disorders.

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