Cell type-specific expression of SEPT3-homology subgroup members controls the subunit number of heteromeric septin complexes

Figure S1 (related to Figure 1): Blue Native PAGE analysis of recombinant septin complexes

(A) Depiction of recombinant hetero-hexamers (1) and hetero-tetramers (2). The subunit arrangement is according to the hexamer that forms in bacteria co-expressing SEPT2, SEPT6, and SEPT7 (PDB ID 2QAG Sirajuddin et al., 2007). The SEPT2 homo-dimer (3) that forms in bacteria expressing SEPT2 alone is also depicted (PDB ID 2QA5).

(B) K562 cells were transfected with the indicated combination of shuttle vectors directing inducible expression of SEPT2 and SEPT6 and counter-selected with hygromycin. High-level expression was induced for 8 h, which generates thick ectopic filaments (Sellin et al., 2011a). SEPT2 and/or SEPT6 containing ectopic filaments are comparably stable but disassemble into soluble components at high ionic strength, which was exploited for selective extraction of assembly-competent recombinant septins (see SUPPLEMENTAL MATERIALS AND METHODS). SEPT2 and SEPT6 were analyzed by Blue Native PAGE combined with immunodetection as in Figure 1.

Interpretation of data in Figure S1:
Septins in mammals comprise 13 paralogs classified into four homology-based subgroups (depicted in Figure 1A). Characterization of recombinant septin complexes has suggested subgroup-restricted pairing preferences among SEPT2 and SEPT6 subgroup members, and that SEPT7—the sole member of its subgroup—is essential for assembly of hexameric complexes (Kinoshita, 2003). These findings were subsequently corroborated and extended by studies of subgroup interdependencies in human cells, which formed the basis of a generic model for how septin hetero-oligomerization is subgroup-directed into hetero-hexamers and -octamers (Sellin et al., 2011b).

Studies of bacterially expressed septins have demonstrated that SEPT2 expressed alone forms dimers, while co-expression with SEPT6 results in hetero-oligomerization (Sheffield et al., 2003; Sirajuddin et al., 2007). The Blue Native PAGE analysis in Figure S1B is consistent with these reports, and defines the migration of the specified dimeric and tetrameric septin complexes, which are plotted in Figure 1C along with native hexamers and octamers.

X-ray crystallography has shown that an N-C interface mediates SEPT2-SEPT2 contact at the center of the hexameric unit that assembles in bacteria co-expressing SEPT2, SEPT6, and SEPT7 (Figure S1A, Sirajuddin et al., 2007). In contrast, SEPT2 expressed alone exists as G-interface linked homo-dimers. At low ionic strength, these stable homo-dimers polymerize through reversible N-C interface interactions. This founding work explains how available hetero-oligomerization partners may direct septin interaction-interfaces.

Under conditions of artificial expression in mammalian cells, both SEPT2 and SEPT6 have a propensity to form ectopic filaments, which appear as thick fibers that are distinct from native septin assemblies (Sellin et al., 2011a). Such ectopic filaments appear to be comparably stable and our protocol to enrich for assembly-competent recombinant SEPT2 and/or SEPT6 proteins relied on high-salt extraction of lysed cells. It is evident from the data in Figure S1B that not only SEPT2, but also overexpressed SEPT6 may form stable homo-dimers that assemble into
ectopic filaments. It should be noted, however, that SEPT2 and SEPT6 dimers appear to be rare, or absent, under native conditions (Figure 3A and 5A). These findings have repercussions for the interpretation of reports based on artificially expressed septins.

**The SEPT9 isoform-specific N-terminal extension confers a disproportionate increase in hydrodynamic volume of both monomers and hetero-octamers**

Table SI (related to Figures 1, 3, & 5): Hydrodynamic parameters of monomeric SEPT9 isoforms as analyzed in crude extracts of SEPT7-depleted cells

| Isoform | Gel filtration (nm) | Sedimentation (S) | Estimated Mass (kDa) | Monomer Mass (kDa) |
|---------|---------------------|-------------------|----------------------|--------------------|
| SEPT9(a) | 5.9                 | 2.9               | 72                   | 65                 |
| SEPT9(b) | 5.9                 | 2.9               | 72                   | 65                 |
| SEPT9(f) | 3.2                 | 3.0               | 40                   | 39                 |

* Estimated mass based on the Stoke radius (nm) and sedimentation coefficient (S).

# Monomer mass deduced from the primary amino acid sequence.

Table SI, experimental procedures: Cells were transfected with a replicating shRNA<sup>SEPT7</sup> vector, which depletes SEPT7 by RNA interference (> 90% depletion, same protocol as in Figures 3E and 5C). Hydrodynamic parameters were determined by gel filtration chromatography and density gradient centrifugation using previously described protocols adapted for analysis of the assembly state of individual septins in crude extracts (Sellin et al., 2011b). Molecular masses were calculated based on the estimated Stoke radius and sedimentation coefficients (Siegel and Monty, 1966). The data are representative for analysis of K562 cells, as well as Jurkat, that are depleted of SEPT7.

Figure S2 (related to Figure 1 and Figure 4-7): The significance of the size of the SEPT9 N-terminal extension for migration of heteromers and monomers separated by Blue Native PAGE.

(A) Depiction of SEPT9(a), SEPT9(e) and SEPT9(f) isoforms, which only differ in the length of the N-terminal extension. Note that K562 cells do not express detectable amounts of endogenous SEPT9(e) (see Figure 1A, which depicts isoforms expressed in K562).

(B) K562 cells were transfected with empty vector, the replicating RNA-interference vector shRNA<sup>SEPT9</sup>, or co-transfected with shRNA<sup>SEPT9</sup> and the indicated pMEP-SEPT9 derivative, which contains silent mutations within the
shRNA\textsuperscript{SEPT9}-targeted sequence. Cells were subsequently counter-selected with hygromycin as in Figure 1, by which homogenous transfected cell populations can be obtained within a week. Septin heteromers were resolved by Blue Native PAGE and transferred to PVDF filters for immunodetection of SEPT9. Complexes in Vector-Co cells corresponding to endogenous hetero-octamers (8-mer) are denoted \textit{i}, \textit{ii}, and \textit{iii} according to premises defined in Figure 1. The overexposures reveal an additional complex (marked by \#) in SEPT9(e) and SEPT9(f) expressing cells. The position of monomers of the indicated SEPT9 isoforms is indicated by arrows.

\textbf{(C)} The relationship between molecular masses and the mobility by Blue Native PAGE of either monomers of the indicated ectopic SEPT9 isoform (symbols marked by*) or hetero-octamers containing the cognate isoforms. The deduced molecular masses of SEPT9 isoform monomers (see panel A) and hetero-octamers were calculated according to premises given in Figure 1. The minor SEPT9(e) and SEPT9(f) complexes (marked by \# on overexposed autoradiograms), which migrate below the 6-mer marker, were also detected by antibodies against SEPT7 as well as SEPT2 and SEPT6 subgroup representatives (unpublished data). This provided the basis for the assumption that these have a molecular mass equivalent to half an octameric building block (symbols marked by \#, depicted molecular masses: (SEPT9(e) hetero-tetramer, 188.5 kDa; SEPT9(f) hetero-tetramer: 179.6 kDa Migration of hexamers (\textbullet, set as 0), tetramers (\textcircled{○}) and dimers (\textDelta) is based on data shown in Figure 1 and S1. The migration of molecular mass markers is also shown (albumin, 67 kDa; ferritin, 450 kDa; dimers of ferritin, 900 kDa). The data presented are representative of at least five independent analyses by Blue Native PAGE.

\textbf{Interpretation of data in Table S1 and Figure S2:}

SEPT7-depletion of K562 and Jurkat cells results in the corresponding depletion of hetero-hexamers and -octamers, which is associated with an altered assembly state and reduced amounts of all the endogenous septin paralogs (Sellin \textit{et al.}, 2011b). This supports the idea that SEPT7 is an obligatory subunit of hetero-hexamers and -octamers, and that hetero-oligomerization with SEPT7 increases the stability of other septins.

The calculated molecular masses of SEPT9 isoforms present in SEPT7-depleted cells deviate by \textasciitilde10\% or less from the deduced masses of monomers (Table 1). Hence, it is clear from these data that endogenous SEPT9 exists as monomers in the absence of SEPT7. In contrast, SEPT2 and SEPT6 subgroup members exist as dimers and/or tetramers in SEPT7-depleted cells, which has been previously shown by determination of hydrodynamic parameters (Sellin \textit{et al.}, 2011b) and corroborated by Blue Native PAGE analysis in the present study (see Figure 3E).

The variable and extended N-terminus is unique to certain SEPT9 isoforms. Table 1 shows that this extension confers a disproportionally large increase in Stoke radius of SEPT9(a) and SEPT9(b) monomers relative to the increment in mass. Such hydrodynamic properties are hallmarks of unfolded and/or intrinsically unstructured proteins (reviewed by Uversky, 2002). Given that the N-terminal extension only comprises \textasciitilde40\% of the total mass of SEPT9(a) and SEPT9(b), the present data suggest that the extension has a very low degree of tertiary structure as compared to the compact structure of the non-variable G-domain.

Figure S2 shows the SEPT9 assembly state in K562 cells in which the endogenous SEPT9 isoforms are replaced by single isoforms (same strategy as in Figure 1B). The expression levels were attuned to achieve stochiometric excess over the endogenous septin paralogs, which results in the appearance of monomeric SEPT9. The analysis included SEPT9 isoforms depicted in Figure S2A, which differ solely by the length of their N-terminal extension. Blue Native PAGE separation of cell extracts combined with SEPT9-detection revealed a disproportionate influence of the N-terminal extension on the migration of both monomers and hetero-octamers (Figure S2B).

Both molecular mass and shape govern separation by Blue Native PAGE. The migration of octamers and monomers plotted against their molecular masses is shown in Figure S2C along with that for dimers, tetramers, and hexamers (defined as in Figure 1 and S1). The data based on the SEPT9(f) isoform reveal a log-linear relationship with the subunit number of septin complexes, which covers a range from 39 kDa up to 359 kDa. Compared to globular proteins with compact structures, septin complexes migrate somewhat more slowly than predicted by their sizes, but the slope is still similar (compare solid and dashed lines). However, the slope of the plotted data (migration \textit{versus} molecular mass) of SEPT9 isoform monomers, as well as of the cognate octamers, is notably different. Thus, it is evident that the N-terminal extensions of these isoforms retard the migration much more than anticipated by the increment of molecular mass.

The combined data in Table 1 and Figure S2 indicate that a large hydrodynamic volume retards the migration of complexes resolved by Blue Native PAGE. It is notable that octamers in SEPT9(e)-transfected K562 cells migrate close to the endogenous complex \textit{i}. However, K562 only expresses SEPT9 isoforms a, b, and f (Sellin \textit{et al.}, 2012) and our evidence suggests that the endogenous complex \textit{ii} consists of octamers containing SEPT9(f) at
one end and SEPT9(a), or SEPT9(b), at the other (Figure 1). Since the N-terminal extension of SEPT9(e) is about half the length of the extensions of SEPT9(a) and SEPT9(b), it appears that half of the extension located at both ends retards octamer migration to a similar extent as the complete extension located at a single end.

The overexposed autoradiograms in Figure S2B confirm that excessive SEPT9 levels primarily result in assembly of octamers, but a minor unique complex migrating below the 6-mer marker was evident in SEPT9(e) and SEPT9(f) overexpressing cells (marked by #). These unique complexes were also visualized by detection of SEPT7, SEPT2 and SEPT6 (unpublished observations), which suggest the presence of hetero-tetramers composed of septin subunits from each one of the four homology subgroups. This interpretation is generally consistent with the migration of the minor complexes in SEPT9(e) and SEPT9(f) expressing cells plotted against the molecular masses of atypical tetramers predicted to be arranged as one half of a hetero-octamer (symbols marked by #). The significance of these results is related to the present identification of this category of atypical tetramers in lymphocytes and brain tissues (see Figure 4 – 7).

**Evidence that hetero-octamers are stably assembled protein complexes**

**Figure S3 (related to Figure 2): The time course of hetero-oligomerization of newly synthesized AcGFP-SEPT9(f) as determined by octamer binding to artificially bundled microtubules**

(A) K562 cells (cell cycle time: ~20 h) were transfected with empty vector (Vec-Co) or co-transfected with the RNA-interference vector shRNASEPT9 and the expression vector pMEP-AcGFP-SEPT9(f). After ~1 week of counter-selection in hygromycin (using conditions that support constitutive SEPT9(f) expression as in Figure 1), septin heteromers were analyzed by Blue Native PAGE combined with immuno-detection of SEPT7 and SEPT9. Endogenous hetero-octamers (8-mer) are denoted i, ii, and iii according to premises defined in Figure 1.

(B) AcGFP-SEPT9(f)-specific fluorescence of cells described in panel A was determined by flow cytometry prior to (Live) or after cell permeabilization, as indicated. Cells were either untreated (Co) or pretreated for 3 min with a high (3 μM) taxol concentration (+Tax), which generates microtubule bundles. Bar charts represent the mean ± errors and the data are expressed as percentage of the AcGFP-SEPT9 signal of live cells.

(C) K562 cells were transfected with pMEP-AcGFP-SEPT9(f) and counter-selected with hygromycin under conditions that suppress expression from the hMTIIa promotor. A transient burst of AcGFP-SEPT9(f) expression was induced as described (Sellin et al., 2011a). At the indicated time points, AcGFP-SEPT9(f)-specific fluorescence...
of cells was determined by flow cytometry prior to (Live) and after taxol pretreatment and subsequent permeabilization as in panel B. The graph represents cell-associated fluorescence units.

**D** Same as panel C, but the scale of the x-axis is set to clarify the time course of microtubule bundling-dependent retention of the AcGFP-SEPT9(f) reporter. The graph represents cell-associated fluorescence units of permeabilized cells that were either untreated (Co) or pretreated with taxol (+Tax), as indicated.

The data presented in panel A-D are representative of at least two independent experiments. Experimental details concerning cell permeabilization, analysis of microtubule bundling-dependent cell retention of AcGFP-tagged septins, and flow cytometry have been described (Sellin et al., 2012).

**Interpretation of data in Figure S3:**

Figure 2 of the *Results* section shows the kinetics by which the septin heteromer composition is altered subsequent to a transient burst of SEPT9 expression, which demonstrates that pre-existing hexamers do not serve as assembly intermediates for octamers. However, this type of experiment involves expression levels that cause aberrant homotypic SEPT9 interactions at early time points, which obscure detection of octamers by Blue Native PAGE analysis. This precluded an evaluation at peak expression of potential exchange with SEPT9 subunits of pre-existing octamers.

The data in Figure S3 relate to putative SEPT9 subunit exchange by an alternative approach, which relies on fusion with the fluorescent AcGFP reporter at the N-terminus of SEPT9. This modification has previously been shown not to detectably interfere with the assembly of ectopic SEPT9(f) into hetero-octamers (Sellin et al., 2012). Figure S3A shows Blue Native PAGE analysis of K562 cells in which endogenous SEPT9 isoforms have been replaced by AcGFP-SEPT9(f). Expression was attuned to obtain a predominant pool of hetero-octamers, but still minimizing the fraction of monomeric AcGFP-SEPT9(f). Detection of SEPT7 and SEPT9 confirmed both the efficiency and specificity in AcGFP-tagging of octamers. It is evident from these results that most heteromers consist of AcGFP-SEPT9(f)-tagged octamers, which due to the AcGFP-fusion partner migrate slightly higher than complex *iii*.

The experimental approach to detect hetero-oligomerization of AcGFP-SEPT9 into functional octamers was based on the propensity of septin heteromers to associate to artificial microtubule bundles (Sellin et al., 2011a; Sellin et al., 2012). Such bundles are generated by treating cells with high concentrations of the microtubule-stabilizing drug taxol. Determination of cell-associated fluorescence intensity in Figure S3B showed that 25–30% of the AcGFP-SEPT9(f) reporter was retained by permeabilized cells that were pretreated for 3 min with taxol, in contrast to non-treated cells which do not retain the reporter. Given the predominance of AcGFP-SEPT9(f)-tagged octamers and that the fraction of monomeric reporter was below the level of detection, as shown in Figure S3A, these data provide an estimate of the fraction of heteromers specifically retained by microtubule bundles under those particular experimental conditions. It should be noted that retention is sensitive to the ionic strength of the permeabilization buffer (Sellin et al., 2012), which suggests that septin heteromer binding to microtubule involves electrostatic interactions, which can be expected to gain in avidity by multiple-bond interactions on bundles.

The data in Figure S3A and B set the premises for our approach to addressing the question of whether octamers exchange their SEPT9 subunits in a way to associate to artificial microtubule bundles. The fluorescence intensity of live cells in Figure S3C indicate a burst of AcGFP-SEPT9(f) expression followed by a decline, which replicates the corresponding data on native SEPT9(f) in the *Results* section (Figure 2) and confirms that optimal stability of AcGFP-SEPT9(f) is dependent on hetero-oligomerization. Moreover, the results show undetectable specific retention at time points at which cells contain high levels of newly synthesized AcGFP-SEPT9(f), which implies that AcGFP-SEPT9(f) monomers do not bind microtubule bundles (compare Figure S3C and D). Importantly, it was also apparent that the fraction of retained AcGFP-SEPT9(f) gradually increased to a level approaching 25% after 72 h. The x-axis in Figure S3D is set to detail reporter retention in taxol-pretreated cells and untreated cells, which establishes a critical dependence on microtubule bundling.

The time course of this experiment corresponds to ~3.6 cell divisions (the cell cycle time of K562 cells is ~20 h), which would mean a ~12-fold dilution of pre-existing heteromers. Thus, the assembly of AcGFP-SEPT9(f)-tagged octamers depends on newly synthesized hetero-oligomerization partners and our data rule out a significant rate of SEPT9 subunit exchange among octamers. This is in apparent contrast to the subunit exchange found between the cognate complexes of budding yeast (McMurray and Thorner, 2008), but is still consistent with the evidence that mammalian hetero-hexamers do not exchange any of their septin subunits (Sellin et al., 2011a). Thus, while properties of septin hetero-oligomers appear to differ between organisms, our studies suggest that both hetero-hexamers and -octamers in mammalian cells are irreversibly assembled and are stable protein complexes.
Tetramers in lymphocytes contain representatives of each one of the four septin subgroups

Figure S4 (related to Figure 4): The subunit composition of septin hetero-tetramers in activated B- and T-lymphocytes

(A) SDS-PAGE and Western blot detection of the indicated septin in lymphocytes. Note that Western blot data on SEPT9 are shown in Figure 4 in the Results section.

(B) and (C) Septin heteromers in B-blasts and primary T-blasts were analyzed by Blue Native PAGE combined with immunodetection of the indicated septin. To visualize SEPT9-containing tetramers, an overexposed autoradiograph is shown. Note that detection of SEPT7 and SEPT9 (moderately exposed) is shown in Figure 4 of the Results section. Septin complexes are annotated according to Figure 1 and the position of atypical heteromers is marked with an arrowhead. The data presented are representative of three independent analyses by Blue Native PAGE.

Interpretation of data in Figure S4:
Figure 4 of the Results section shows that lymphoid cell types contain hetero-tetramers, which migrate slightly more slowly than the recombinant SEPT2-SEPT6 tetramers that define the marker denoted 4-mer (see Figure 1 and S1). Hetero-tetramers are abundant in Jurkat cells and readily detectable by both anti-SEPT7 and anti-SEPT9. However, hetero-tetramers are less abundant in B-blasts and T-blasts and not detected by anti-SEPT9 at the exposures shown in the Results section, which were selected to show the relative proportions of hetero-octamer subsets. At the exposure levels shown in Figure S4, which distort detection of distinct octamer subsets, it becomes clear that detection of SEPT9 reveals hetero-tetramers in B-blasts and T-blasts.

The data in Figure S4B and C include detection of SEPT2 and SEPT6. The corresponding data on Jurkat T-blastoid cells are shown in Figure 5A in the Results section. It is evident from these results that hetero-tetramers in lymphocytes contain members of each one of the four septin homology subgroups.
Blue Native PAGE resolution properties of atypical versus SEPT2-SEPT6 hetero-tetramers

Figure S5 (related to Figure 5): Side-by-side comparisons of heteromers in control Jurkat cells and counterparts expressing shRNA\textsuperscript{SEPT7} and shRNA\textsuperscript{SEPT9}.

The transfected Jurkat cell lines described in Figure 5 were analyzed by Blue Native PAGE combined with immunodetection of SEPT2 (A) and SEPT6 (B). Septin complexes are annotated according to Figure 1 and the position of atypical tetramers is marked with an arrowhead. The data are representative for Jurkat cell lines derived from two independent transfection experiments.

Interpretation of data in Figure S5:
The exposures in Figure S5 are selected to clarify the relationship of tetramers in control and shRNA expressing Jurkat T-blastoid cells. These exposures, combined with running of samples side-by-side, show that tetramer complexes in control Jurkat cells (marked with arrow heads) can be distinguished from the SEPT2-SEPT6 tetramers that accumulate in SEPT7-deficient cells, which as anticipated migrates as the 4-mer marker defined in Figure 1S.

In control Jurkat cells, detection of SEPT6, SEPT7 and SEPT9 visualizes only the tetramer complex marked with arrow heads, but SEPT2-detection visualizes an additional complex migrating as the 4-mer marker (Figure 5 and S5). A cognate SEPT2-detected complex, which is unaffected by SEPT9-depletion, was also evident in K562 cells (Figure 3, 5 and S5). Hence, this complex is not cell type specific.

Animal septins are abundant (at least 0.1 % of all cellular proteins (Sellin et al., 2011b), which facilitate faithful detection of specific septin complexes. Furthermore, native filamentous septin structures disassemble into soluble protomer units upon cell permeabilization in a PEM-buffer which was originally optimized for preservation of tubulin hetero-dimers (Sellin 2011). This provides a gentle protocol for preparing soluble protomer units of native septin filaments (>95 % of all septins are recovered in the soluble fraction). As shown in Figure 4, hexamers and octamers in various cell types are evidently well preserved by the present protocols. We have also found that the proportion of heteromeric septin complexes remains unaltered under conditions of increased ionic strength (NaCl concentrations in the range 0.3 up to 1 M, unpublished observations). Finally, scoring of decay products of native octamers and hexamer after freeze/thawing indicated that heteromer decays is associated with aggregation and did not reveal any septin complexes that may result from fracture of heteromers.

To further address whether potential fracture of octamers may occur in Jurkat cells, separation by Blue Native PAGE were compared with gel filtration, which provides the gentlest means available for size-separation of protein complexes. Gel filtration confirmed that Jurkat cells contain a major fraction of heteromers which are smaller than hexamers and that SEPT9 depletion results in a uniform pool of hexamers (unpublished observations). Hence, detection of atypical tetramers in Jurkat cells cannot be explained by octamer fracture during Blue Native PAGE separation.

The control experiments outlined above support the notion that hetero-tetramers in Jurkat T-blastoid cells represent authentic protomer units of septin filaments. Similar to the cognate hetero-tetramers in primary lymphocytes (Figure 4 and S4), this heteromer category is detected by antibodies against each one of the four homology subgroups. Current evidence suggests that subgroup-restricted subunit arrangement is common to all heteromers. Hence, it seems likely that atypical tetramers are arranged as one half of an octameric building block as depicted in Figure 5D.
Table S2 (related to Figure 6): Predominant septin heteromers in SEPT9(f), SEPT3 and SEPT12 expressing K562 cell lines.

| Transfection*: | Predominant heteromeric complexes** |
|----------------|----------------------------------|
| Vector-Co K562, 8-mer: | 9(a/b/f)-7-6s.gr-2s.gr–2s.gr-6s.gr-7-9(a/b/f) |
| 6-mer: | 7-6s.gr-2s.gr–2s.gr-6s.gr-7 |
| Ectopic SEPT9(f), 8-mer: | 9(f)-7-6s.gr-2s.gr–2s.gr-6s.gr-7-9(f) |
| Ectopic SEPT3, 8-mer: | 3-7-6s.gr-2s.gr–2s.gr-6s.gr-7-3 |
| 4-mer: | 3-7-6s.gr-2s.gr |
| Ectopic SEPT12, 8-mer: | 9(f)-7-6s.gr-2s.gr–2s.gr-6s.gr-7-9(f) |
| 4-mer: | 12-7-6s.gr-2s.gr |

*Transfection conditions as described in Figure 6.
**The arrangement of the indicated septin paralog is depicted according to the same premises as Figure 1A and 3G. The head-to-head arrangement around the central SEPT2s.gr-SEPT2s.gr N-C interface of octamers and hexamers is indicated (black-to-red). SEPT9 isoforms are indicated within brackets. Atypical tetramers are depicted to be arranged according to homology subgroup membership (s.gr).

Characterization of the reporter system used to visualize how SEPT3 subgroup members modulate higher-order filamentous septin structures

FIGURE S6 (related to Figure 6): SEPT7-AcGFP levels of cells expressing the indicated SEPT3 subgroup member.
Cell lines in which endogenous SEPT7 was replaced by SEPT7-AcGFP (described in Figure 6D and E) were analyzed by flow cytometry. The distribution of background (K562 wild type, upper panel) and AcGFP-fluorescence...
among live cells expressing the indicated SEPT3 subgroup member is shown. More than 97% of all cells were included in the acquisition gate and 5000 cells were analyzed.

**Lower Panels:** Cells were stained with propidium iodide followed by analysis of DNA content.

Interpretation of data in Figure S6:
The conclusion based on Figure 6D and E rely on visualization of septin assembles by replacement of endogenous SEPT7 with SEPT7-AcGFP. Figure S6 shows the distribution of AcGFP-fluorescence intensity among live cells, which suggest that the expression levels of the fluorescence reporter are essentially unaltered by ectopic expression of SEPT9(f), SEPT3 or SEPT12. Moreover, the DNA-profiles shown in the lower panels Figure S1, lower panels, show that the expressed SEPT3 subgroup members did not detectably interfere with cell growth and division.

**SUPPLEMENTAL MATERIALS AND METHODS:**

**Enrichment of recombinant septins assembled into ectopic filaments of transfected cells**
Recombinant high-level expression of SEPT2 and/or SEPT6 generates thick ectopic filaments that do not resemble any native septin structures (Sellin et al., 2011a). Ectopic filaments are comparably stable, but de-polymerize at high ionic strength, which was exploited for enrichment of assembly-competent recombinant SEPT2 and SEPT6. Accordingly, transfected K562 cells were resuspended in 80 mM PIPES, pH 6.9, 2 mM MgCl₂, 4 mM EGTA (PEM buffer) containing 0.2% saponin and 10 μg/ml leupeptin, and kept on ice for 5 min. Cell lysis by this protocol disassembles all native septin structures into soluble components, which are released into the cytosolic fraction (Sellin et al., 2011b). The insoluble cell fraction was isolated by centrifugation (0.5 min, 2 000 × g) and subsequently resuspended in a PEM buffer containing 0.5 M NaCl. After 5 min on ice, cell nuclei and insoluble proteins were removed by centrifugation (20 min, 14 000 × g). Proteins in the clarified supernatant, which mainly contained assembly-competent recombinant SEPT2 and SEPT6, were analyzed as described in Figure S1.

**Shuttle vector DNA constructs directing regulatable expression of septins**
The Epstein-Barr virus (EBV-) based episomally replicating pMEP4 shuttle vector was used for experiments involving ectopic expression and gene product exchange. This vector directs expression from the regulatable hMTIIa promoter and confers hygromycin resistance on mammalian cells (Groger et al., 1989). Construction of pMEP derivatives directing expression of SEPT2 and SEPT6 has been described (Sellin et al., 2011b).

The protein isoforms corresponding to SEPT9 transcript variants 1, 2, 5/6, and 7 are termed SEPT9 isoforms a, b, e, and f according to NCBI RefSeq (see Figure 1A). Construction of pMEP derivatives of native and AcGFP-tagged SEPT9(a), SEPT9(e), and SEPT9(f) isoforms has been described previously (Sellin et al., 2012). These were all made resistant to shRNA<sub>SEPT9</sub>-mediated suppression by introducing 4 silent mutations. The cognate pMEP-SEPT9(b) isoform (transcript variant 2) derivative was generated by an analogous strategy. Briefly, the pMEP-SEPT9(a) derivative was used as a PCR-template with a forward primer that replaced the SEPT9(a) unique exon 1 and 3 with the SEPT9(b) unique exon 2, which contains the ATG initiator codon and the first 18 residues of SEPT9(b) as depicted below. The reverse primer covers the unique KpnI site located in an exon encoding the G-domain (common to all confirmed SEPT9 isoforms; 5'-GGTGTTTTTCACTTCGATGGTAC). The PCR fragments were digested with KpnI and inserted into the corresponding site of the shRNA<sub>SEPT9</sub>-resistant cloning intermediate termed pMEP-SEPT9-KpnI-sh616imm (Sellin et al., 2012), which regenerated the native open reading frames of the SEPT9(b) isoform. The coding sequences were confirmed by DNA sequencing.

**SEPT9(b)-specific forward primers:**

\[
\text{KpnI} \quad \text{HindIII} \\
5'-ACTCGGTACCAAGCTTCAACATGTGGACCCCGTGCAACGCGAGCTGGATGGGATCATTT
\]

\[
\text{CGGACTTCCGAGCCCTTGAAGATCTTTGAGGT} \\
\text{KpnI} \quad \text{HindIII} \\
5'-ACTCGGTACCAAGCTTCAACATGTGGACCCCGTGCAACGCGAGCTGGATGGGATCATTT
\]

\[
\text{CGGACTTCCGAGCCCTTGAAGATCTTTGAGGT} \\
\text{KpnI} \quad \text{HindIII} \\
5'-ACTCGGTACCAAGCTTCAACATGTGGACCCCGTGCAACGCGAGCTGGATGGGATCATTT
\]

\[
\text{CGGACTTCCGAGCCCTTGAAGATCTTTGAGGT} \\
\text{KpnI} \quad \text{HindIII} \\
5'-ACTCGGTACCAAGCTTCAACATGTGGACCCCGTGCAACGCGAGCTGGATGGGATCATTT
\]

\[
\text{CGGACTTCCGAGCCCTTGAAGATCTTTGAGGT} \\
\text{KpnI} \quad \text{HindIII} \\
5'-ACTCGGTACCAAGCTTCAACATGTGGACCCCGTGCAACGCGAGCTGGATGGGATCATTT
\]

\[
\text{CGGACTTCCGAGCCCTTGAAGATCTTTGAGGT} \\
\text{KpnI} \quad \text{HindIII} \\
5'-ACTCGGTACCAAGCTTCAACATGTGGACCCCGTGCAACGCGAGCTGGATGGGATCATTT
\]

Nucleotides corresponding to the 5’ untranslated sequence preceding the ATG initiator codon are italicized. The coding sequence of exon 5, which is unique to the SEPT9(b) isoform, is underlined. The subsequent nucleotides are complementary to exon 7, the entire coding sequence of which is shared by the SEPT9 a, b, and c isoforms.

Human SEPT3 (transcript variant B; NM_019106.5) and SEPT12 (transcript variant 1; NM_001154458.2) cDNAs, which were modified to encode an N-terminal Flag epitope tag, were custom-synthesized by GenScript (www.genscript.com). The common 5’ sequence preceding the open reading frames of SEPT3 and SEPT12 contained a HindIII site, a 5’ untranslated sequence preceding the ATG initiator codon of SEPT9(a), and a sequence encoding the eight-residue Flag epitope tag followed by an Neo site (5’-AAGCTTGGAGGCACCATGGACTAC
AAGGACGACGATGAC(AAGGGC). The synthetic genes were inserted into the HindIII and BamHI sites of pMEP4 to generate pMEP-Flag-SEPT3 and pMEP-Flag-SEPT12.

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