Supplemental Figures and Material

Microtubules support a disc-like septin arrangement at the plasma membrane of mammalian cells
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Figure S1

**FIGURE S1: Unbalanced SEPT7 expression causes accumulation of insoluble aggregates.** The experiment was performed as in Figure 1B, but both the soluble and insoluble cellular proteins were analyzed. Thus, the Western blot in panel A corresponds to data in Figure 1B (i.e. soluble proteins), while the Western blot in panel B shows proteins that are insoluble in Triton X-100 lysis buffer and extracted by boiling in SDS-sample buffer. Proteins corresponding to 0.25 x 10^6 cells (~25 μg) were loaded in each lane, resolved by 12 % gradient SDS-PAGE, and analyzed by Western blot using the indicated antibodies (α-tubulin: loading control).

Interpretations:
The data reveal transient accumulation of insoluble His-SEPT7 between 4 to 72 h after the initial burst of expression (the tagged His-SEPT7 migrates above endogenous SEPT7). The disappearance of His-SEPT7 aggregates at 96 hours can be attributed to both degradation and dilution during cell divisions.

Figure S2
FIGURE S2: Mislocalization and formation of ectopic cytosolic assemblies by septin-AcGFP reporters stably expressed in K562 cells. Cell lines expressing the indicated septin AcGFP-reporters were generated by a protocol aimed to optimize hetero-oligomerization and analyzed as described in Figure 3B. A deconvoluted optical section at the equatorial plane is shown at the top and a phase-contrast image at the bottom. The presented images of SEPT2-AcGFP (panel A) and SEPT6-AcGFP (panel B, the cell at the right has gone through “mitotic slippage”; hence its large size) show the most typical ectopic filaments and filament bundles observed in these cell lines. The image of SEPT9-AcGFP (panel C) shows cytosolic localization with a clearing at the position of the nucleus. Scale bar, 5μm.

Interpretations:
SEPT2-AcGFP was found to only partially hetero-oligomerize with endogenous septins (Figure 2C). Moreover, contrary to initial expectations, increasing expression levels actually decrease the fraction of endogenous septins in heteromeric complexes with artificially expressed SEPT2 (unpublished data, which include analysis of both SEPT2-Flag and SEPT2-AcGFP). This may be explained by the propensity of singly expressed SEPT2 to polymerize through alternating G- and NC-interface interactions (Mendoza et al., 2002; Huang et al., 2006; Sirajuddin et al., 2007). Thus, it seems likely that homotypic interactions of overexpressed SEPT2 contribute to both poor hetero-oligomerization and ectopic structures formed by SEPT2-AcGFP.

SEPT6-AcGFP competes successfully with all three endogenous SEPT6-subgroup members expressed by K562 cells and hetero-oligomerizes quite efficiently (Figure 2A and D). This implies that the subunit-composition of the AcGFP-tagged heteromer pools will not correspond to native heteromers. In addition, the AcGFP-reporter at the C-terminus may interfere with native higher-order assemblies. Finally, the ~25 % fraction of non-heteromeric SEPT6-AcGFP may poison authentic septin assemblies (Figure 2D). Hence, there are three possible explanations for the observed mislocalization of the SEPT6-AcGFP reporter.

The cytosolic localization of SEPT9-AcGFP is evident in all cells and we did not note any ectopic filaments or aggregates. Mislocalization of this reporter was indeed expected since it completely fails to hetero-oligomerize with endogenous septins (Figure 2F). This failure can be attributed to fusion of AcGFP to the C-terminus (unpublished data). It should be noted that members of the SEPT3-subgroup – SEPT3, SEPT9 and SEPT12 – lack the entire C-terminal coiled-coil extension that is a hallmark of other septins. This may explain why a C-terminal fusion partner blocks heteromer assembly of SEPT9, but not SEPT2, SEPT6 and SEPT7.

We noted a profound heterogeneity among SEPT2- and SEPT6-AcGFP expressing cells. Both of these overexpressed septins localized diffusely throughout the cytoplasm as well as to a variety of ectopic structures in the cytosol. The appearance of these structures seem to correlate with expression levels and, in cells with the most intense fluorescence, large cytosolic aggregates were also evident (unpublished data). The heterogeneity made it difficult to pin-point specific differences between these reporters. Even so, while SEPT6-AcGFP appeared to essentially fail to visualize authentic septin assemblies, in occasional low expressing SEPT2-AcGFP cells we noted a similar localization as endogenous septins (Figure 3A), but the fluorescence was low and the specificity unsatisfactory (unpublished data).
Figure S3: Filaments formed by non-heteromeric SEPT2 or SEPT6 sequester endogenous core heteromers and thereby interfere with native cortical assemblies. By using the regulatable expression system described in Figure 1, transfected K562 cells were induced to express the indicated AcGFP-reporter for a limited time period (14 hours), which implies an essentially non-heteromeric state (see Figure 1D). Cells were subsequently fixed and immuno-stained with anti-SEPT7 combined with a red fluorescence DyLight549 labeled secondary antibody. Deconvoluted optical sections at the equatorial plane are presented. Non-stained AcGFP expressing cells confirmed undetectable bleeding of AcGFP fluorescence into the red channel (not shown). Left panels show the localization of endogenous SEPT7 (red fluorescence) in Vector-Co and the indicated septin-AcGFP transfected cells. Middle panels show the localization of either SEPT2- or SEPT6-AcGFP (green fluorescence). Right panels show an overlay of red and green fluorescence (yellow indicates coinciding signals). Scale bar, 5µm.

Interpretations:
The data in Figure 1B – D suggest that the core heteromer assembly process is co-translational and irreversible. This notion is also supported by our previous study on the temporal order of heteromer assembly (Sellin et al., 2011). Accordingly, as shown by sedimentation analysis of lysates from His-SEPT7 transfected cells in Figure 1D, fourteen hours of induced expression of a septin-reporter is too short to achieve a significant degree of hetero-oligomerization with endogenous septins. This assumption was confirmed by sedimentation analysis of cell lysates of cells analyzed in Figure S3, which revealed that <5 % of SEPT2-AcGFP and SEPT6-AcGFP were assembled into heteromers. It follows that the ectopic filaments in Figure S3 consist of non-heteromeric AcGFP-reporters.

Given that non-heteromeric SEPT2 or SEPT6 polymerize into cytosolic structures that colocalize with endogenous SEPT7, the present data suggest that ectopic filaments nucleated by a single septin have the potential to incorporate endogenous septin core heteromers. Consistent with this interpretation, it is also evident that the native cortical septin assemblies (as immuno-detected by anti-SEPT7 in Vector-Co cells) are depleted in cells expressing SEPT2-AcGFP or SEPT6-AcGFP. These results indicate that artificially expressed SEPT2- or SEPT6-subgroup septins may exert dominant negative effects.

GENERAL DISCUSSION OF DATA PRESENTED IN FIGURES S2 AND S3
The present evaluation of reporter-systems for imaging of septins in human cells provides examples of non-native structures caused by unbalanced expression of individual septins. Indeed, only the SEPT7-AcGFP reporter provided faithful visualization of endogenous septins, but also in this case it is important to optimize conditions for complete assembly into septin core heteromers. Our findings summarized in Figure 1, 2, S2, and S3 have implications for the interpretation of published work that relies on septin reporters; in particular since some of these reports have inferred individual septins as important for diverse cellular functions.
It is notable that the predominant ectopic structures formed by artificially expressed SEPT2 or SEPT6 all reside in the cytosol, while native septin assemblies detected in K562 and Jurkat cells are all plasma membrane associated. Surprisingly, ectopic structures are formed irrespectively of heteromeric context (compare Figures S2 and S3). Thus, if expressed at non-physiological levels, both SEPT2 and SEPT6 seem promiscuous with respect to oligomerization/polymerization partners and the resulting ectopic structures do not exhibit the plasma membrane binding propensity of native septins. It is notable that the ectopic structures visualized by SEPT2-AcGFP or SEPT6-AcGFP in K562 cells resemble structures reported in mammalian cells transfected with a variety of tagged septin-derivatives.

Many studies of septin localization have been based on SEPT2-reporters containing a fluorescent protein fused at its C-terminal, i.e., the same principle design as SEPT2-AcGFP used in this study. Although we noted localization to apparently native septin assemblies in sporadic low-level expressing cells, ectopic filaments were a prevalent feature. Our results seem consistent with the propensity of recombinant SEPT2 to polymerize into filaments and bundles in a low salt buffer (Mendoza et al., 2002; Huang et al., 2006). The structure of these filaments, as well as the hexameric SEPT2/6/7 heteromer, has been solved by X-ray crystallography (Sirajuddin et al., 2007). It was shown that, while an NC-interface mediates SEPT2-SEPT2 contact in the center of the SEPT2/6/7 hexamer, SEPT2 expressed alone forms stable G-interface linked homodimers, which in turn polymerize through the NC-interface.

There is no evidence that G-interface linked SEPT2 homodimers exist in normal cells. At least in K562, Jurkat and HeLa cells, it appears that endogenous septins exist solely in the context of 6- to 8-subunit core heteromers (Sellin et al., 2011). Nonetheless – despite conditions aimed to optimize hetero-oligomerization – we still found that the bulk of SEPT2-AcGFP, as well as SEPT2-Flag, exist as stable homodimers (Figure 2C and unpublished data). Thus, artificial SEPT2 expression appears to favor formation of stable G-interface connected SEPT2 homodimers, which are evidently hetero-oligomerization incompetent and have an innate propensity to form extended filaments.
FIGURE S4: Uniformly-sized $\Phi \sim 0.8 \, \mu m$ disc-like arrangements at the cell cortex are the predominant interphase septin assemblies detected in non-adhered spherical K562 cells. The SEPT7-AcGFP expressing K562 cell line is described in Figures 2E and 3. (A) A fluorescence intensity profile across a disc-like septin structure. The deconvoluted optical section (parallel to the plasma membrane) that was used for scanning is shown below (arrows indicate the scanned section). (B) Deconvoluted optical sections (1.5 $\mu m$ apart) of a representative interphase SEPT7-AcGFP expressing cell are shown. (C) 51 consecutive optical sections of 10 representative interphase SEPT7-AcGFP expressing cells were used for determining the number of disc-like septin structures (CellR, Olympus software). The cell surface area was calculated based on the diameters of each cell (15 $\mu m$ fluorescent beads were used for calibration). (D) Deconvoluted optical sections at the equatorial plane of a methanol-fixed cell are shown. Left panel show the localization of SEPT7-AcGFP (green fluorescence), middle panel show immuno-stained microtubules (red fluorescence). Right panel show an overlay of red and green fluorescence. The cell cycle stage of the presented cell corresponds to late metaphase/early anaphase. Scale bars, 5$\mu m$.

Interpretations:
K562 cells are of hematopoietic origin, grow in suspension, and do not depend on substrate-adhesion for survival. The data in Figure 3 and S4 serve to characterize septin discs, which are the predominant higher-order filamentous septin arrangement during the interphase of the cell cycle of non-adherent cells. It is also shown that septins do not detectably localize to the mitotic spindle of K562 cells and remain at the cortex during metaphase and anaphase of mitosis (Figure S4D). However, septin assemblies during metaphase do not appear as uniformly punctuate as in interphase cells. These observations seem consistent with septin filaments being rearranged prior to cytokinesis.

FIGURE S5: Actin filaments are not essential for microtubule-supported septin assemblies in K562 cells. The SEPT7-AcGFP expressing K562 cell line is described in Figures 2E and 3 and deconvoluted optical sections at the equatorial plane are shown. (A) Cells were paraformaldehyde-fixed and filamentous actin was detected by Alexa Fluor568-labelled phalloidin. Left panel shows the localization of SEPT7-AcGFP (green fluorescence), middle panel shows localization of phalloidin (red fluorescence). Right panel shows an overlay of red and green fluorescence. (B) Cells were treated for 60 min with the actin filament-disrupting drug cytochalasin D (2 $\mu g/ml$) followed by fixation and phalloidin staining as in panel A. The data show that septin assemblies remain intact (green), while the drug efficiently disrupts cortical actin filaments (red, note that the cytosolic actin structures remain but have an altered appearance). (C) Deconvoluted optical sections at either the equatorial plane or parallel to the plasma membrane of
cytochalasin D treated cells prior to fixation. The data show that the disc-like structures with a central clearing, which are visible in optical sections parallel to the plasma membrane of living cells, appear unaltered by depolymerization of cortical actin filaments (compare with the corresponding imaged of untreated live cells in Figure 3B). Scale bar, 5μm.

Interpretations:
The observed localization of filamentous actin in K562 cells, namely to the cortex and in cytosolic structures surrounding the Golgi apparatus, is consistent with other reports in which this cell line has been studied, e.g. (Preisinger and Kolch, 2010). Figure S5B shows that cytochalasin D treatment efficiently disperses cortical filamentous actin and it appears that the cytosolic structures stained by phalloidin increase in abundance. Even so, under these conditions all detectable septin assemblies remain at the cortex. We have observed SEPT7-AcGFP expressing K562 cells for up to 4 h in the presence of cytochalasin D, but still not noted any signs of dispersed septin discs in interphase K562 cells. Thus, cortical actin filaments are not of significance for the disc-like septin arrangement.

Figure S6

A

| shRNA-Op18 | - | + | - | - |
| shRNA-MAP4 | - | - | + | - |
| shRNA-SEPT7 | - | - | - | + |

Blot:

- Op18
- MAP4
- SEPT2
- SEPT6
- SEPT7
- SEPT9<sup>75kDa</sup>
- Tubulin

B

![Graph showing % polymeric tubulin](image)

FIGURE S6: Endogenous Op18/Stathmin and MAP4 mediate readily detectable counteractive regulation of microtubule content. K562 cells were transfected with the indicated shRNA vector and counter-selected with hygromycin for one week as in Figure 5A. Cellular proteins (A) and the partitioning of tubulin heterodimers (B) were analyzed as in Figure 4B. Data are plotted as percentage polymeric tubulin of the estimated total content of polymerization-competent tubulin.
Interpretations:
The results show that depletion of either of two established counteractive microtubule regulatory proteins have clear-cut effects on the microtubule content of K562 cells. In contrast, depletion of all septins (by mean of shRNA-SEPT7), has no detectable effects. We were also unable to detect any effects of septin depletion by the appearance of the microtubule-array in fixed cells (unpublished data).

Figure S7

| Op18-Flag: | - | + | - | - |
| MAP4-Flag: | - | - | + | - |
| SEPT2-Flag: | - | - | - | + |

Overexpression (fold): ~6 ~8 ~17

FIGURE S7: Excessive overexpression of SEPT2 does not cause detectable alterations in microtubule content. K562 cells were transfected with EBV-based replicating vectors directing inducible expression of Op18/Stathmin, MAP4 or SEPT2. Transfected cell lines were counter-selected with hygromycin for one week and protein expression was induced for 8 hours as in Figure 5C. The partitioning of tubulin heterodimers was analyzed as in Figure 4B. Quantification of endogenous and overexpressed Op18/Stathmin, MAP4 and SEPT2 proteins after 8 hours of induced expression in K562 cells was achieved by serial dilution of total lysates and comparison with a standard curve of recombinant proteins (Holmfeldt et al., 2002; Sellin et al., 2008; Sellin et al., 2011).

Interpretations:
This experiment shows that ~6-fold increased levels of the microtubule-destabilizing Op18/Stathmin protein cause a dramatic decrease of microtubule content, while an ~8-fold increase of the microtubule stabilizing protein MAP4 has the expected opposite effect. However, even a ~17-fold increase of SEPT2 has no detectable global effects that alter the partitioning of tubulin heterodimers.

GENERAL DISCUSSION OF DATA PRESENTED IN FIGURES S6 AND S7

Septins have previously been proposed to modulate interphase microtubules by both globally and locally acting mechanisms. These include (i) sequestering of the microtubule stabilizing protein MAP4 (Kremer et al., 2005), (ii) binding to a microtubule subset and thereby specifying polarized transport in epithelial cells (Spiliotis et al., 2008) and (iii) serving as filamentous guidance tracks for a microtubule subpopulation during epithelial cell polarization (Bowen et al., 2011). The data in Figures S6 and S7 make it very unlikely that septins exert global regulation of microtubules in K562 cells through MAP4 sequestering. In addition, by pull-down experiments using Flag-tagged MAP4, SEPT6, SEPT7, we have been unable to reproduce the proposed interactions between MAP4 and septins (unpublished data).

Our experiments do not address the proposed localized actions of septins that are specific for polarizing epithelial cells (Spiliotis et al., 2008)(Bowen et al., 2011). Nor does the data exclude local effects on microtubules in K562 cells. Nevertheless, our data on microtubule content are consistent with a study in which artificially expressed borg3 was shown to potently sequester septins in epithelial cells without detectable effects on the appearance of the interphase microtubule-array (Joberty et al., 2001).
FIGURE S8: Septins bind to microtubule bundles that gradually form in taxol treated cells. SEPT7-AcGFP expressing K562 cells were cultured with taxol (3 μM) for 4 hours as in Figure 6. Cells were thereafter fixed and immunostained with anti-α-tubulin combined with a red fluorescence labeled secondary antibody. Deconvoluted optical sections at the equatorial plane of two adjacent cells are shown. Left panels show the localization of septin core heteromers (as visualized by SEPT7-AcGFP, green fluorescence). Middle panel show the localization of microtubules (red fluorescence). Right panel show an overlay of red and green fluorescence (yellow indicates coinciding signals). Cell outlines are shown in white. Scale bar, 5μm.

Interpretations:
The result confirms that the elongated cytosolic septin structures that accumulate over time in taxol-treated cells can be attributed to binding onto microtubule bundles. Similarly to septin binding to actin bundles (Kinoshita et al., 2002), microtubule-binding may be indirect and depend on some unknown adaptor. As shown in Figure 3, septins do not bind detectably to individual microtubules, which suggests that bundling is required to provide sufficient binding cooperativity. The significance of microtubule-bundling for septin-binding is consistent with the localization of septins during remodeling of the microtubule system that occur in polarizing epithelial cells (Bowen et al., 2011).

FIGURE S9: Each one of SEPT2, SEPT6 and SEPT9 are dispensable for microtubule-dependent preservation of septin assemblies in permeabilized cells. K562 cells were transfected with the indicated shRNA vector and counter-selected with hygromycin for one week as in Figure 5A. Panel A shows the degree of shRNA mediated depletion of the targeted septin protein (α-tubulin: loading control). Panel B shows the partition of septin core heteromers (as defined by SEPT7) after permeabilization by saponin as in Figure 7B. The data show the partitioning in the presence of microtubule-stabilizing taxol (3 min, 3 μM) or microtubule-destabilizing nocodazole (3 min, 6 μM). The released (Supernatant, S) and cell-associated (Pellet, P) proteins were analyzed by Western blot detection of SEPT7 and α-tubulin.
Interpretations:
Individual septins have often been attributed specific properties, which includes microtubule-binding. In particular SEPT9 has been implicated as a microtubule interacting septin, but these studies did not consider the heteromeric context of SEPT9 (Surka et al., 2002; Nagata et al., 2003). By depletion of SEPT2, SEPT6, or SEPT9, we do not note that any of these individual septins are of particular significance for the specific link between septins and microtubules addressed in Figure S9, namely preservation of insoluble septin assemblies in permeabilized cells.

Nocodazole was used to obtain complete microtubule depolymerization in the experiment shown in Figure S9. However, it is evident from Figure 7B that essentially all septins are also released into the soluble fraction after permeabilization of untreated cells, which still contain some residual microtubules.

Our previous study suggests that septins exist solely in the context of a repertoire of 6 to 8 subunits core heteromers, which are assembled according to homology subgroups rather than individual members. Consequently, in most cases the heteromers are not arranged as perfect palindromes (Sellin et al., 2011). To set the premises for characterization of the complete protomer unit pool in that study, septin partitioning between soluble and particulate assembly states were evaluated at various ionic strengths. This did not reveal any differences in the proportional distribution of individual septins between these two states. Thus, both microtubule-supported septin assemblies, as shown in Figures 7C and S9, and septin assemblies preserved by low ionic strength, appear the same with respect to equal representation of the individual septins. Thus, despite the diversity among individual core heteromers, our previous and present analyses suggest that the entire heteromer repertoire contribute roughly equally to the insoluble septin assemblies of K562 cells.

Figure S10

FIGURE S10: Analysis of permeabilized Jurkat cells shows that microtubule stabilization preserves septin assemblies. The data show the partitioning of septins in Jurkat cells permeabilized as in Figure 7B in the presence of microtubule-stabilizing taxol (3 min, 3 μM) or microtubule-destabilizing nocodazole (3 min, 6 μM). The released (Supernatant, S) and cell-associated (Pellet, P) proteins were analyzed by Western blot detection of representatives of each of the four septin subgroups. The cytosolic Op18 protein serves as control for complete permeabilization in the presence of taxol.

Interpretations:
Consistent with the analysis of K562 cells in Figure 7B and C, the data in Figure S10 suggest that septins representing each one of the four homology-based subgroups are equally represented in the cortical septin discs detected in Jurkat cells.
**Figure S11**

**FIGURE S11:** K562-KA8 cells that spread on an adhesion-substrate contain heterogeneously sized and shaped septin structures with a central clearing. SEPT7-AcGFP expressing K562-KA8 cells were plated onto an invasin-coated surface as in Figure 9. A deconvoluted optical section parallel to the attachment substrate is shown. Scale bar, 5 μm. Arrow heads indicate the largest and smallest circular structure with a clear-cut central clearing.

**Interpretations:**
The image shown in Figure S11 is chosen to illustrate that spreading of K562-KA8 cells transforms the comparatively uniform-sized septin discs (see Figure 3B-C) into a wide variety of structures present all over the attachment-substrate. These include heterogeneously sized and shaped structures displaying a central clearing, which suggest a potential for septins to form a variety of closed structures.

**SUPPLEMENTAL MATERIAL:**

**Construction of AcGFP and DsRed reporter derivatives**

To construct pMEP4 and pCEP4 shuttle vectors directing expression of septin-AcGFP reporters, intermediate derivatives encompassing the complete open reading frames of each septin followed by HindIII / NotI restriction sites were generated by PCR. This allowed in-frame insertion of the AcGFP-reporter excised as a 740 nt by HindIII / NotI fragment from pAcGFP1-N1 (Cat. No. 632469, Clontech Laboratories, Mountain View, CA). The template and primers used to create the intermediate SEPT2 derivative were as follows:

**SEPT2 (361 aa, NCBI Reference Sequence: NP_001008492.1)**

5’-primer: 5’-GACCGCAACCCAGCTGGTCGCTTGTCTTCTATTACCATGTCTAAGCAACAGCCAACTC

3’-primer: 5’-CCGCGGAACTAAGCGGCCGCCTGGCCTGAAAGCTTCACGTGGTGCCCGAGAGCCCCGC

Other septin AcGFP reporters used in this study were constructed by analogous PCR strategies based on the following templates: SEPT6 isoform A (427 aa, NCBI Reference Sequence: NP_665799.1), SEPT7 isoform 1 (437 aa, NCBI Reference Sequence: NP_001779.3), SEPT9 isoform A (586 aa, NCBI Reference Sequence: NP_001106963.1). The coding sequences of all PCR-generated fragments were confirmed by nucleotide sequence analysis.

To construct the pMEP-Lifeact-DsRed reporter derivative, the 17 amino acid actin filament binding peptide (Riedl et al., 2008) together with a Gly-Gly-Ser-Gly linker was fused to the N-terminus of DsRed-Monomer by a PCR strategy. The template and primers were as follows:

**DsRed-Monomer-N1 (Cat. No. 632465, Clontech Laboratories, Mountain View, CA).**

5’-primer: 5’-GGACCCAAGCTTGCCTTTGCTTTCTATTCACC ATGGGCGTGGCTGACCTTATTAAAAAGTTCGAGTCTATTTCTACAGGAGGAAGGCCGCTCAGG GTCGCCACCATGGACACACC

3’-primer: 5’-GATTATGATCTAGAGTCGCGGC

The PCR fragment was digested with HindIII and NotI and used to replace the corresponding fragment of the pMEP4 vector.
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