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Requirements of Fission Yeast Septins for Complex Formation, Localization, and Function[D]

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Septins are GTP binding proteins important for cytokinesis in many eukaryotes. The *Schizosaccaromyces pombe* genome sequence predicts orthologues of four of five *Saccharomyces cerevisiae* septins involved in cytokinesis and these are named Spns1-4p. That *spns1-4* are not essential genes permitted the application of a combined genetic and proteomics approach to determine their functional relationships. Our findings indicate that Spns1-4p are present throughout interphase as a diffusely localized ~8.5S complex containing two copies of each septin linked together as a chain in the order Spn3p-Spn4p-Spn1p-Spn2p. Septin recruitment to the medial region of the cell is genetically separable from ring formation, and whereas it is normally restricted to mitosis, it can be promoted without activation of the mitotic cell cycle machinery. Coalescence into ring structures requires Spn1p and Spn4p associate with at least one other septin subunit and the expression of Mid2p that is normally restricted to mitosis. This study establishes the functional requirements for septin complex organization in vivo.

INTRODUCTION

Septins are a family of guanine nucleotide binding proteins. They were first identified in *Saccharomyces cerevisiae* for their role in cytokinesis (Hartwell, 1971). In the past 10 years, it has become evident that septins are highly conserved cytoskeletal elements found in most eukaryotes (Faty *et al.*, 2002; Macara *et al.*, 2002). Although many septins have been implicated in the process of cell division because of their localization to the cell division site and/or because the disruption of their function leads to cytokinesis defects, septins are likely involved in additional biological processes because they are present in nondividing cells such as neurons and in complexes with components of the secretory apparatus (reviewed in Field and Kellogg, 1999; Kartmann and Roth, 2001; Faty *et al.*, 2002).

Septins typically exist in heterooligomeric complexes (Field *et al.*, 1996; Frazier *et al.*, 1998; Hsu *et al.*, 1998; Sheffield *et al.*, 2003). In *Drosophila*, three septins form a stable complex that likely consists of a linear array of each septin homodimer (Field *et al.*, 1996). When expressed in *Escherichia coli*, three mammalian septins can form a complex of a size consistent with a hexamer, i.e., two of each subunit (Sheffield *et al.*, 2003). In *S. cerevisiae*, four septins form a stable complex that has been predicted to consist of two molecules each of Cdc3p, Cdc10p, and Cdc12p and 1 molecule of Cdc11p (Frazier *et al.*, 1998). Although clearly existing in complexes, the organization of septins within these complexes has not been investigated previously.

In *S. cerevisiae*, Cdc3p, Cdc10p, Cdc11p, and Cdc12p together with another septin, Shs1/Sep7, (Mino *et al.*, 1998) assemble into a patch at the incipient bud site during G1. As the cell cycle progresses, septins reorganize at the mother-bud neck into an hourglass-shaped collar of cortical filaments (Frazier *et al.*, 1998). Recombinant septins have the capacity to form filaments in vitro in a GTP-dependent manner (Mendoza *et al.*, 2002; Versele and Thorner, 2004). However, although GTP-binding is important for in vitro filament assembly and ring formation in vivo (Versele and Thorner, 2004), nucleotide turnover on septins in vivo is so slow that GTP hydrolysis is unlikely to play a role in septin dynamics during the cell cycle (Vrabioiu *et al.*, 2004). Rather, other events regulated by the GIN4 family of kinases (Longtine *et al.*, 1998; Mortensen *et al.*, 2002; Dobbelaere *et al.*, 2003) and/or Cla4p (Dobbelaere *et al.*, 2003; Schmidt *et al.*, 2003; Versele and Thorner, 2004) in *S. cerevisiae* have been shown to be important for assembly of functional septin rings in vivo. However, exactly how septin complexes are assembled into higher order structures such as rings remains unknown.

Once organized into a ring structure in *S. cerevisiae*, septins are thought to perform multiple functions at the mother-daughter bud neck. These include providing a boundary that restricts certain determinants to particular cortical domains (reviewed by Faty *et al.*, 2002) and acting as a bud neck scaffold necessary for the localization of many factors involved in polarity and cell division (reviewed by Gladfelter *et al.*, 2001). Whether septins perform all of these roles in other organisms is yet to be established.

Septins have a conserved structure. They contain a central GTP-binding domain flanked by a basic region at the amino terminus, and most septins contain a coiled-coil domain at the carboxy terminus. With the completion of the *Schizosaccharomyces pombe* genome sequence (Wood *et al.*, 2002), ho-

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mologues of the *S. cerevisiae* septins Cdc3p, Cdc10p, Cdc11p, and Cdc12p were identified and named Spn1p, Spn2p, Spn3p, and Spn4p, respectively. *spn1-4* are not essential, but the absence of any or all of them (Berlin *et al.*, 2003; Tasto *et al.*, 2003; our unpublished data) causes a delay in the completion of cell division, resulting in a chained cell phenotype. Spn1p and Spn3p are known to organize at the site of cell division late in mitosis where they form a ring that splits as the septum forms, but this ring does not constrict with actomyosin ring invagination; rather, the ring dissipates after cell cleavage (Berlin *et al.*, 2003; Tasto *et al.*, 2003).

Mid2p is a *S. pombe* protein related at its C-terminus to *S. cerevisiae* Bud4p, *Candida albicans* Int1p, and anillins present in multicellular eukaryotes (Field and Alberts, 1995; Sanders and Herskowitz, 1996; Oegema *et al.*, 2000; Gale *et al.*, 2001). Anillin is able to direct septin filament assembly along actin bundles in an in vitro assay containing only purified components (Kinoshita *et al.*, 2002). Mid2p lacks an obvious F-actin binding domain as is present in anillin, but Mid2p is similarly required for the proper organization of septin rings at the site of cell division (Berlin *et al.*, 2003; Tasto *et al.*, 2003). Like septins, *mid2*$^+$ is not an essential gene, but its loss causes a chained cell phenotype (Berlin *et al.*, 2003; Tasto *et al.*, 2003). Fluorescence recovery after photobleaching (FRAP) analysis of Spn1p-GFP has shown that septin rings are quite dynamic in *mid2*Δ cells, whereas in wild-type cells they are very stable (Berlin *et al.*, 2003). Mid2p production is normally restricted to anaphase and overproduction of a stabilized truncation mutant of Mid2p leads to persistence of septin rings through multiple cell divisions (Tasto *et al.*, 2003). These observations suggest that Mid2p interacts either directly or indirectly with septins to promote their organization into stable ring structures in late mitosis.

To begin to address how Mid2p and its relatives affect septin ring organization, we have investigated how Spns 1-4p are organized in *S. pombe* cells using a combination of proteomic and genetic approaches. Our data suggest that the building blocks of septin rings are ~8.5S complexes containing two of each septin subunit linked together in an almost linear array of specific order. These units are assembled into a functional ring structure in at least two steps. Recruitment to the medial region can occur if septation is forced during interphase but coalescence into a ring structure requires Mid2p that is normally produced only in mitosis. Our data also indicate that each septin subunit contributes uniquely to the formation of septin structures.

MATERIALS AND METHODS

Yeast Strains, Media, and Genetic Methods

S. pombe strains used in this study are listed in Supplementary Table 1 and were grown in YE or minimal medium with the appropriate supplements as described (Moreno *et al.*, 1991). DNA transformations were done by electroporation (Prentice, 1992) or lithium acetate transformation (Keeney and Boeke, 1994). To arrest cells in S phase, cultures were grown in 12 mM hydroxyurea at 25°C for 4 h.

The *spn1*, *spn2*, *spn3*, and *spn4* open reading frames (ORFs) were each tagged at their 3′ ends with the *HA3-Kan*^{R}, *myc13-Kan*^{R}, *2X CTAP-Kan*^{R}, *CFP-Kan*^{R}, *YFP-Kan*^{R}, and *EGFP-Kan*^{R} cassette as previously described (Bahler *et al.*, 1998). Kan^R transformants were screened by whole cell PCR and then by immunoblotting to confirm the accurate integration and expression of the fusion protein.

The *spn1* ORF was replaced with *ura4*$^+$ by homologous recombination in a diploid strain (Bahler *et al.*, 1998). Ura$^+$ transformants were screened for the proper gene disruptant by whole-cell PCR. A heterozygous diploid strain was then sporulated at 25°C followed by tetrad dissection to isolate the haploid *spn1:: ura4* strain.

Table 1. TAP/DALPC results

	Strains							
	1	2	3	4	5	6	7	8
Protein								
Spn1p	14	15	17	12	0	0	16	15
Spn2p	14	12	12	0	0	0	13	11
Spn3p	14	13	15	11	17	13	0	0
Spn4p	10	10	13	9	12	0	10	0

Strains: 1) *spn3-TAP*, 2) *spn4-TAP*, 3) *spn2-TAP*, 4) *spn3-TAP spn2*Δ, 5) *spn3-TAP spn1*Δ, 6) *spn3-TAP spn4*Δ, 7) *spn4-TAP spn3*Δ, *and 8) spn2-TAP spn4*Δ. Numbers in columns below the line are unique tryptic peptides identified from each protein by mass spectrometry.

Molecular Biology Techniques

PCR amplifications were carried out with TaqPlus Precision polymerase (Stratagene, La Jolla, CA) according to manufacturer's instructions. Oligonucleotides were synthesized by Integrated DNA Technologies, Inc. (Coralville, IA).

Purification and Analyses of Septin TAP Complexes

Eight-liter cultures of strains producing TAP-tagged proteins were grown to log phase and the tagged proteins were isolated as described (Tasto *et al.*, 2001). For silver staining, one quarter of the eluate from the purification was precipitated with TCA, resuspended in LDS-PAGE buffer and resolved on a 4–12% NuPAGE using MOPS buffer (Novex, Encinitas, CA). Silver staining was carried out using the Plus One kit as recommended by the manufacturer (Amersham Pharmacia Biotech, Piscataway, NJ). The TAP complexes were analyzed by DALPC-tandem mass spectrometry as described previously (Ohi *et al.*, 2002; Sanders *et al.*, 2002).

Immunoprecipitations, Sucrose Gradients, and Immunoblots

Whole cell lysates were prepared in NP-40 buffer followed by anti-HA or anti-Myc immunoprecipitations as previously described (McDonald *et al.*, 1999). Denatured lysates were prepared as described (Burns *et al.*, 2002). For sucrose gradient analysis, a 200-μl volume of protein lysate was layered onto a 5-ml 10–30% sucrose gradient prepared in NP-40 buffer. Gradients were ultracentrifuged at 38,000 rpm for 18 h in a Beckman SW50.1 rotor (Berkeley, CA). Sedimentation markers were fractionated on gradients prepared and spun in parallel. Fractions were collected from the bottom of the gradient and resolved on 4–12% NuPAGE gels in MOPS buffer and subsequently transferred by electroblotting to PVDF membrane (Immobilon P; Millipore, Bedford, MA). Immunoblotting was done with anti-HA (12CA5; 2 μg/ml), anti-Myc (9E10; 2 μg/ml), or anti-GFP (1:1000 dilution of serum) antibodies. Primary antibodies were detected with horseradish peroxidase–conjugated goat anti-mouse or goat anti-rabbit secondary antibodies (0.4 mg/ml; Jackson ImmunoResearch Laboratories, West Grove, PA) at a dilution of 1:50,000 followed by ECL visualization using SuperSignal (Pierce, Rockford, IL).

Yeast Two-hybrid Assays

The yeast two-hybrid system used in this study was described previously (James *et al.*, 1996). *spn3* and *spn1* cDNAs were cloned into the bait plasmid pGBT9 (Clontech, Palo Alto, CA), *spn1*$^+$, *spn2*$^+$, *spn3*$^+$, *and spn4*$^+$ cDNAs were cloned into the prey plasmid pGAD424 (Clontech) and sequenced to ensure the absence of PCR-induced mutations and that the correct reading frame had been retained.

To test for protein interactions, both bait and prey plasmids were contransformed into *S. cerevisiae* strain PJ69-4A. β-galactosidase reporter enzyme activity in the two-hybrid strains was measured using the Galacto-StarTM chemiluminescent reporter assay system according to the manufacturer's instructions (Tropix, Bedford, MA) with the exception that cells were lysed by glass bead disruption. Each sample was measured in triplicate. Reporter assays were recorded either on the BMG luminometer (Bartlett-Williams Scientific, Chapel Hill, NC) or the Mediator Phl luminometer (Aureon Biosystems, Vienna, Austria).

Microscopy

Strains producing GFP-, YFP-, or CFP-tagged proteins were grown in YE medium and visualized in live cells or in cells fixed with ethanol. Microscopy was performed at room temperature using a spinning disk confocal microscope (Ultraview LCI; PerkinElmer, Norwalk, CT) and Ultraview LCI software (v5.2; PerkinElmer) for image acquisition. Images were processed using

Velocity software (v1.4.2; Improvision, Lexington, MA). Z-series optical sections were taken at 0.5-μm spacing for 3D reconstructions.

RESULTS

The Four S. pombe *Septins Form a Linear Complex*

To define the building blocks of *S. pombe* septin rings, we analyzed how *S. pombe* Spn1p, Spn2p, Spn3p, and Spn4p interacted. For these experiments, *spn1-4* were tagged at their endogenous loci with sequences encoding a variety of epitopes (TAP, HA, myc, GFP, CFP, and/or YFP) to create C-terminal–tagged variants under control of their own promoters. Unless otherwise noted, only strains that exhibited wild-type phenotypes were used for these studies. *spn1-4* were also deleted from the genome either alone or in combination. Although none of these *S. pombe* proteins is essential, the absence of any or all of them causes a delay in the completion of cell division resulting in a chained cell phenotype, although the severity of this defect varies (Berlin *et al.*, 2003; Tasto *et al.*, 2003; see below and our unpublished observations).

First, we examined the composition of Spn3p-TAP, Spn4p-TAP, and Spn2p-TAP complexes purified from asynchronous populations of cells. The *spn1-TAP* strain was not used because it displayed a loss of function phenotype (unpublished data). Spns 1-4p were detected by silver staining and mass spectrometry in all three purifications (Figure 1, A–C and Table 1). No additional proteins were identified by mass spectrometry as specific components of these septin complexes. All other identified proteins were detected in mock TAP eluates from strains lacking TAP-tagged proteins and/or from unrelated TAP complexes (unpublished data).

Next, the composition of septin TAP complexes purified from strains lacking one of the septin proteins was determined by silver staining and mass spectrometry. In Spn3-TAP complexes purified from *spn2*Δ cells, Spn3p, Spn4p, and Spn1p were identified (Figure 1D and Table 1). In Spn3-TAP complexes purified from *spn1*Δ cells, only Spn3p and Spn4p were present (Figure 1E and Table 1). Although Spn2p did not copurify with Spn3p from *spn1*Δ cells, Spn2p-GFP was readily detected in an *spn1*Δ strain suggesting that it is not degraded in the absence of Spn1p (Figure 1I). Spn3p-TAP did not copurify with any other septin from an *spn4*Δ strain (Figure 1F and Table 1). These data suggested that septins interacted in a linear order of Spn3p-Spn4p-Spn1p-Spn2p (see Figure 2A). To confirm this, we examined the composition of the Spn4p-TAP complexes from *spn3*Δ cells and Spn2p-TAP complexes from *spn4*Δ cells. As predicted, Spn4p, Spn1p, and Spn2p were detected in Spn4p-TAP complexes from *spn3*Δ cells (Figure 1G and Table 1) and in the Spn2p-TAP complex isolated from *spn4*Δ cells, Spn1p and Spn2p but not Spn3p were detected (Figure 1H and Table 1). Spn3p was present, however, in cells lacking Spn4p, so degradation did not explain the lack its association with other septins (Figure 1I). Finally, we examined whether Spn4p and Spn1p remained associated in the absence of both Spn2p and Spn3p. Consistent with the proposed Spn3p-Spn4p-Spn1p-Spn2p arrangement, Spn1p-myc and Spn4p-HA coimmunoprecipitated from lysates prepared from a *spn2*Δ *spn3*Δ strain (Figure 1J). Based on the lower recovery of coimmunoprecipitated proteins, this Spn1p-Spn4p complex appears to be less stable than in the presence of Spn2p or Spn3p. Consistent with the coimmunoprecipitation results, Spn4p-TAP complexes purified from *spn2*Δ*spn3*Δ cells contained Spn1p as determined by mass spectrometry as well as significantly more heat shock proteins than the TAP complexes described above (unpublished data). We interpret this as an indicator of complex instability and/or aggregation.

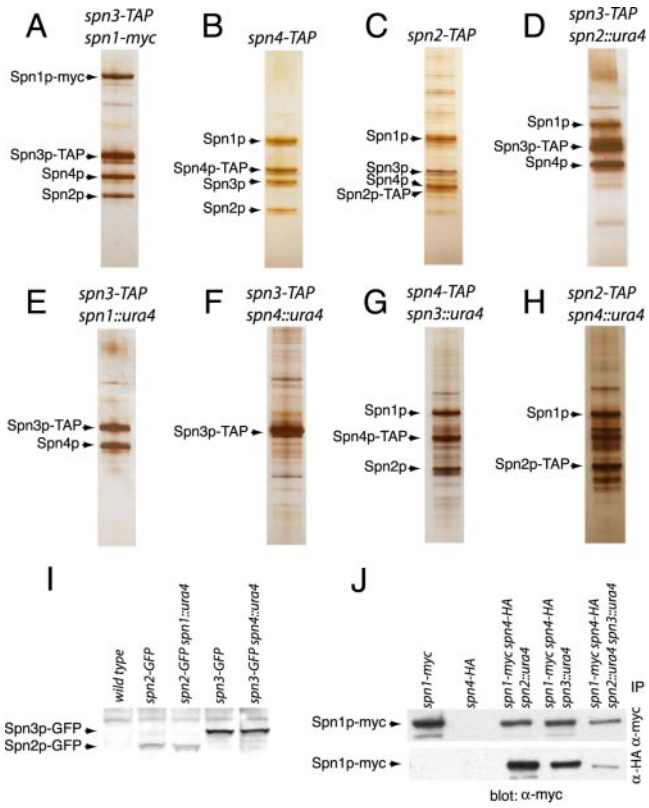

Figure 1. Composition of *S. pombe* septin complexes. (A–H) Silver-stained gels of a portion of Spn-TAP complexes from the indicated strains. Spn proteins are indicated with arrows. Note that the calmodulin-binding portion of the TAP tag remains after the purification and changes the mass of the proteins. Other bands are nonspecific contaminants. (I) Protein lysates prepared in denaturing conditions from the indicated strains were resolved by SDS-PAGE and blotted with anti-GFP serum. (J) anti-myc (top panel) and anti-HA (bottom panel) immunoprecipitates from the indicated strains were blotted with anti-myc antibodies.

To examine the ability of septins to interact with each other in a different manner, directed two-hybrid analysis was performed. As would be predicted by a linear model of interaction derived from the TAP experiments (Figure 2A), Spn3p interacted robustly with Spn4p, but not with Spn1p or Spn2p (Figure 2, B and C). Also, Spn1p interacted with both Spn2p and Spn4p, but not Spn3p (Figure 2, D and E). We next addressed the final possibility of whether Spn2p could interact with Spn4p. Inconsistent with the linear model of assembly derived from the TAP experiments (Figure 2A), significant interaction was observed between Spn2p and Spn4p (Figure 2, F and G). Although we considered the possibility that this interaction might be bridged by a *S. cerevisiae* septin, this seems unlikely because we did not detect any other bridging interactions in our analysis (for example, *spn2* with *spn3*, or *spn3* with *spn1*). Therefore, we conclude that most likely 1) Spn2p can interact directly with Spn4p and 2) Spn1p is required to stabilize this association during a biochemical purification of the complex. Thus, our model was refined by these experiments to encompass the likelihood of a Spn2p-Spn4p interaction stabilized by Spn2p-Spn1p and Spn4p-Spn1p associations (Figure 2H).

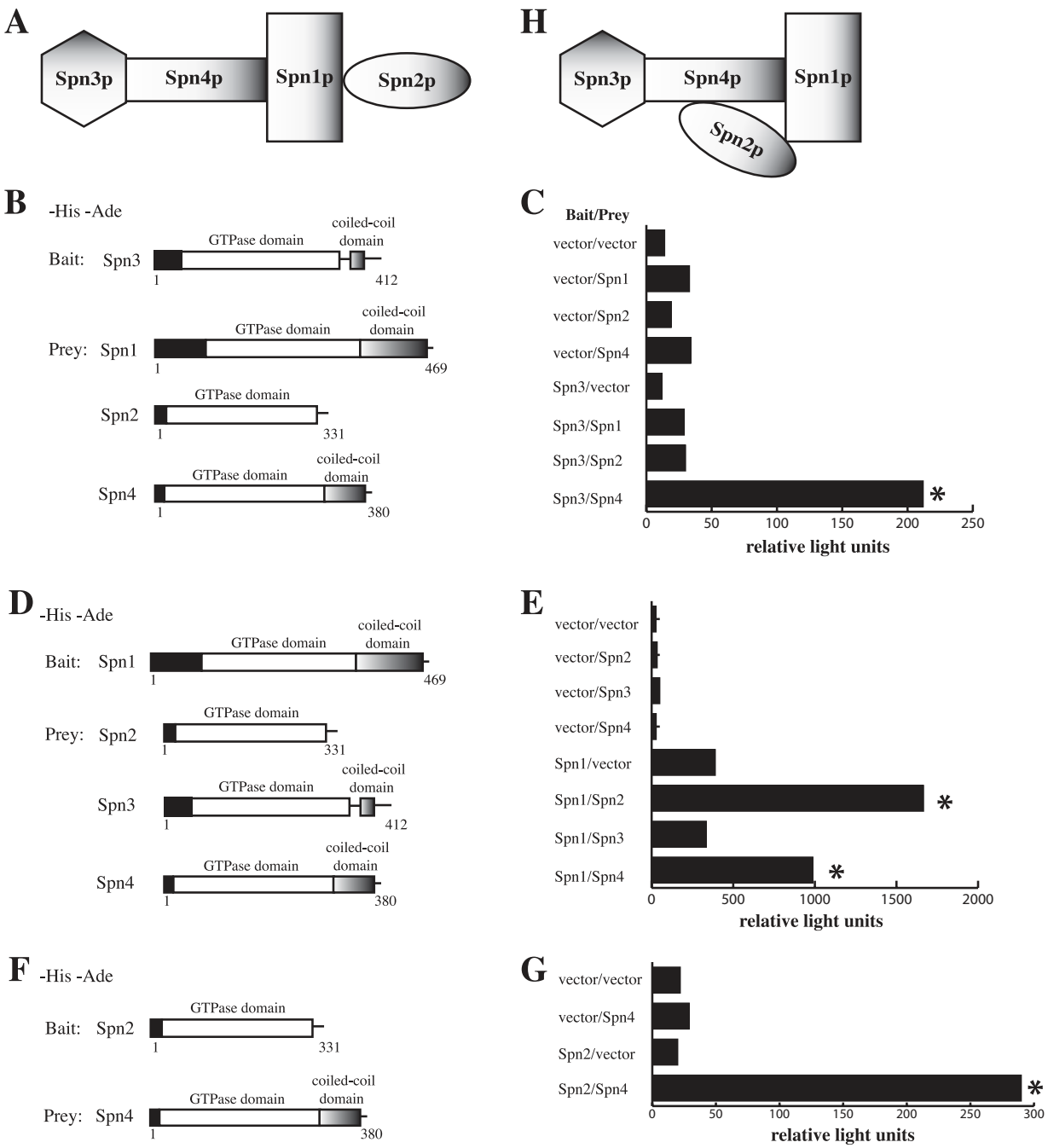

Figure 2. Protein-protein interactions mapped between septin complex components. (A and H) Models of *S. pombe* septin interactions. (B) The PJ69-4A strain was transformed with pGBT9 or pGBT9*spn3*, and pGAD424 or pGAD424 carrying *spn1*, *spn2*, or *spn4*, which are shown schematically. (C) β-galactosidase activity (represented in relative light units) of the stains described in B. (D) The PJ69-4A stain was transformed with pGBT9 or pGBT9*spn1* and pGAD424 or pGAD424 carrying *spn2*, *spn3*, or *spn4*, which are shown schematically. (E) β-galactosidase activity (represented in relative light units) of the stains described in D. (F) The PJ69-4A stain was transformed with pGBT9 or pGBT9*spn2* and pGAD424 or pGAD424 carrying *spn4*, which are shown schematically. (G) β-galactosidase activity (represented in relative light units) of the stains described in D. Asterisk indicates positive interactions.

S. pombe *Septin Complexes Contain at Least Two Copies of Each Subunit*

We next asked whether each septin protein existed primarily in a complex with other septins by sucrose gradient analysis. Each myc- or HA-epitope–tagged septin present in lysates from asynchronously growing cells migrated with a single peak of 8.5S (Figure 3A), indicating that the majority of each septin exists primarily as a component of a larger septin complex. Because septin rings would be expected to sediment much faster than 8.5S, we reasoned that the 8.5S complex corresponds to the septin ring organizational unit present throughout the remainder of the cell cycle and does not correspond to higher order ring structures that must disassemble under our lysis conditions. Indeed, Spn4p-myc

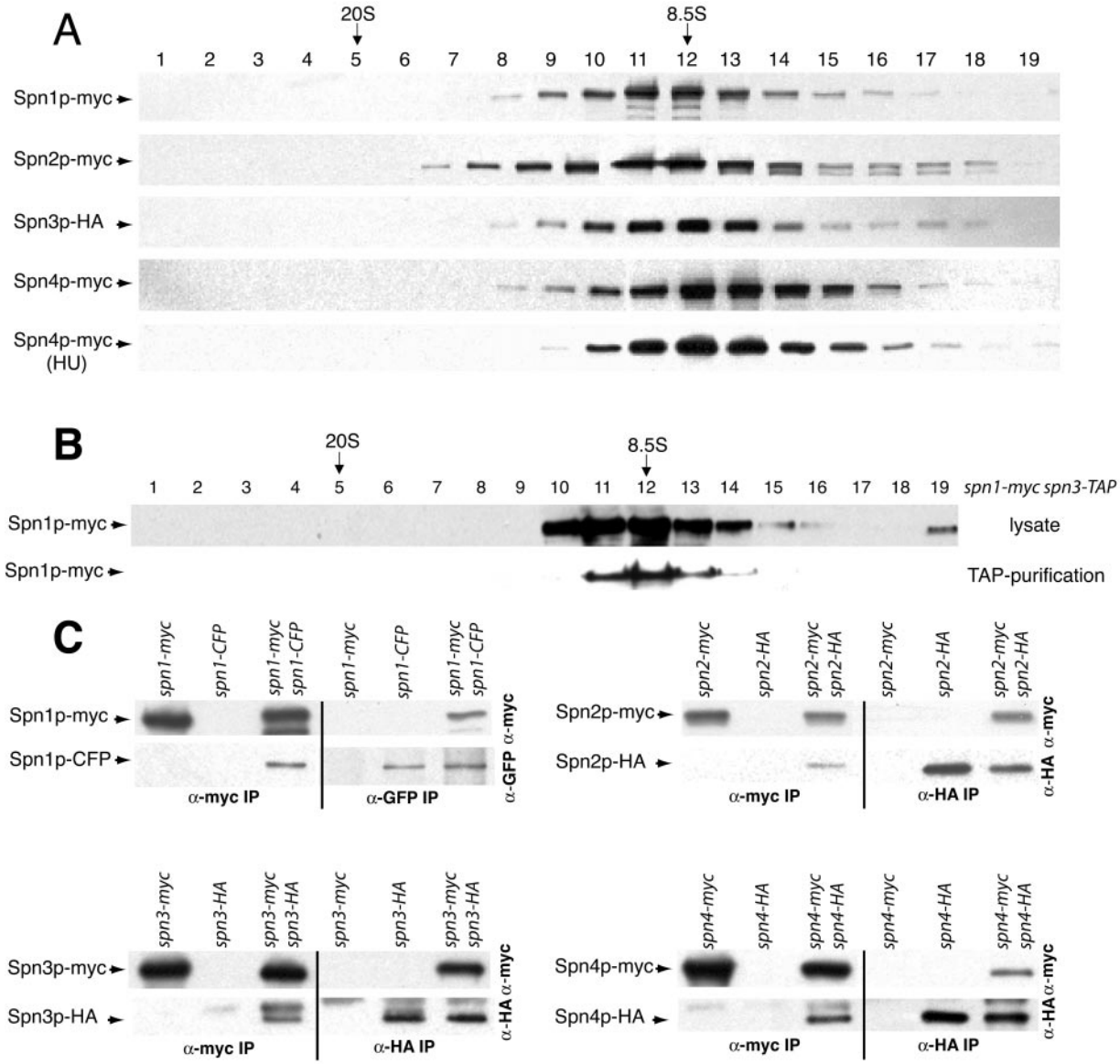

Figure 3. Characterization of *S. pombe* septin complexes. (A) Lysates prepared in NP-40 buffer from each myc-epitope– or HA-epitope–tagged septin protein from asynchronously growing cells or a hydroxyurea (HU)-arrested culture were resolved on sucrose gradients. Fractions were collected from the bottom of the gradient (1) and immunoblotted with 9E10 or 12CA5 to detect myc or HA-tagged proteins. The peaks of thyroglobulin (20S) and aldolase (8.5S) collected from gradients prepared and run in parallel are indicated. (B) A TAP and a lysate prepared in NP-40 buffer from *spn1-myc spn3-TAP* cells were resolved on sucrose gradients. Fractions were collected from the bottom of the gradient and immunoblotted with 9E10 to detect Spn1p-myc. The peaks of thyroglobulin (20S) and aldolase (8.5S) collected from gradients prepared and run in parallel are indicated. (C) anti-myc (left side of panels) and anti-GFP or anti-HA (right side of panels) immunoprecipitates from the indicated strains were blotted with anti-myc (top panels) and anti-GFP or anti-HA (bottom panels) antibodies.

was found primarily in the 8.5S fraction in hydroxyurea-treated cells (Figure 3A) that are arrested in S-phase (unpublished data). We have so far been unable to purify a larger complex containing septins (unpublished data). We also confirmed that the 8.5S complex is what is purified by tandem affinity purification because Spn1p-myc purified by tandem affinity purification from a *spn1-myc spn3-TAP* strain cosedimented with unpurified Spn1p-myc from a protein lysate (Figure 3B).

The sedimentation value of the *S. pombe* septin complex at 8.5S is similar to that reported for a *S. cerevisiae* septin complex (9S), which is predicted to contain two each of three subunits and one of a fourth (Frazier *et al.*, 1998). To determine if *S. pombe* septins are organized similarly, diploid strains containing two different epitope-tagged alleles of each septin were constructed. These diploid strains were phenotypically wild-type (unpublished data) but the two epitope tags could introduce some instability to the complex. In the cases of *spn2-HA/spn2-myc*, *spn3-HA/spn3-myc*, and *spn4-HA/spn4-myc* strains, anti-HA immunoprecipitates contained the myc-tagged septins in addition to the HA-tagged septins (Figure 3C). Coprecipitating proteins were not observed in the single HA-tagged strains (Figure 3C). Conversely, anti-myc immunoprecipitates from the double-tagged strains but not the single myc–tagged strains contained HA-tagged proteins in addition to myc-tagged pro-

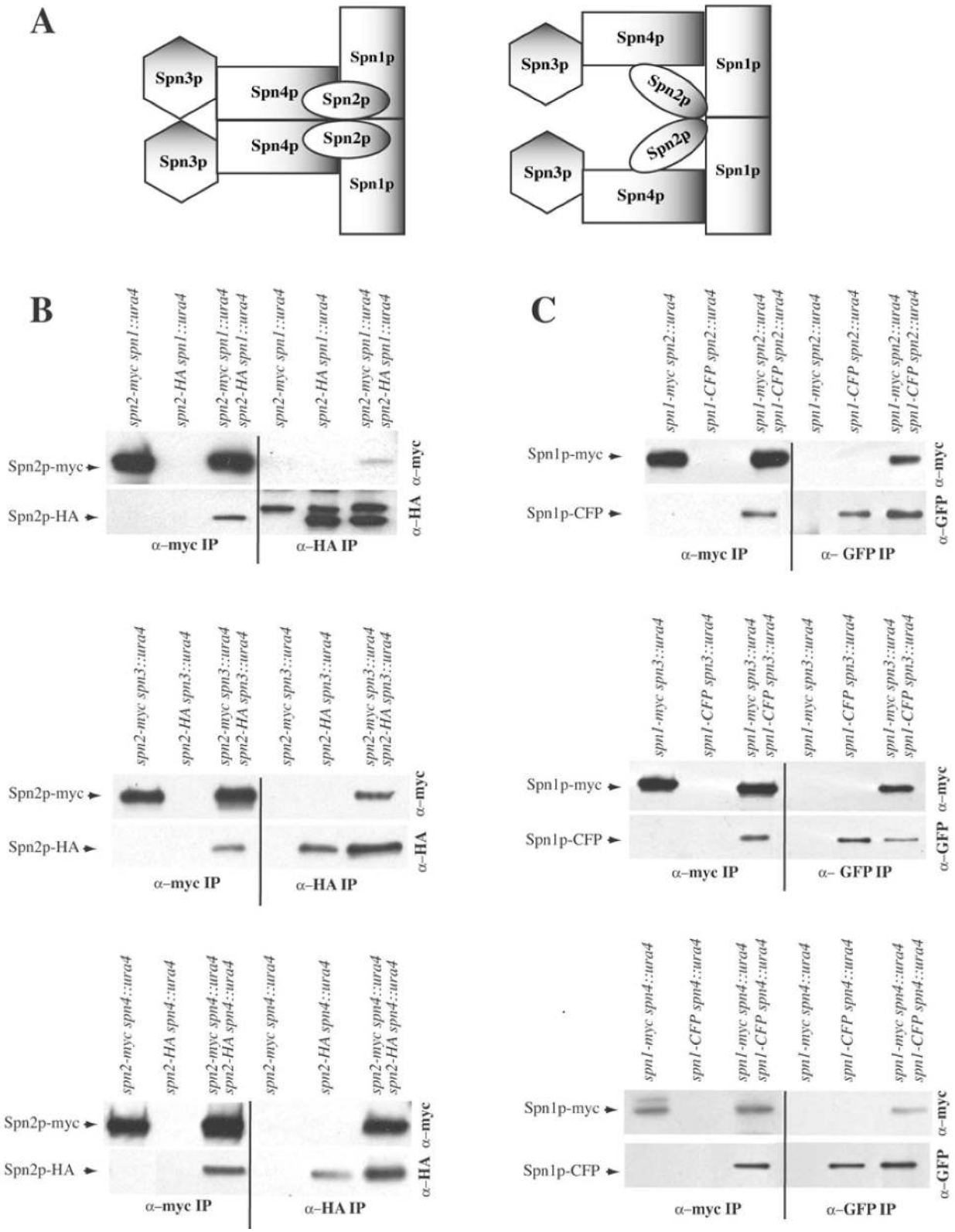

Figure 4.

teins. In lysates prepared from *spn1-CFP/spn1-myc* diploid cells, antibodies to GFP precipitated both Spn1p-CFP and Spn1p-myc and conversely anti-myc immunoprecipitations contained both Spn1p-myc and Spn1p-CFP. Coimmunoprecipitation was not observed in strains producing single epitope–tagged proteins (Figure 3C). From these data, we

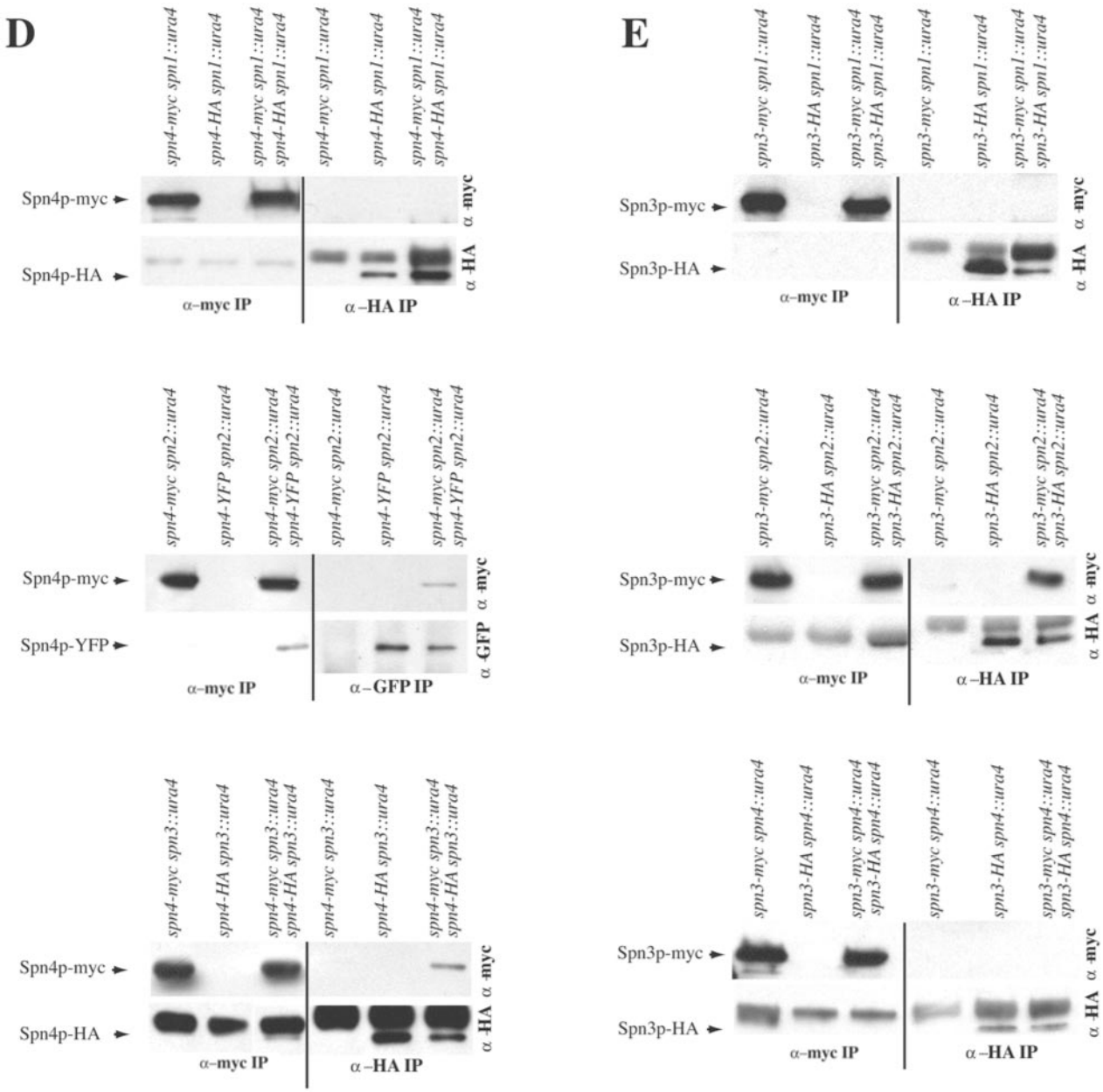

Figure 4 (facing page). *S. pombe* septins interact in cooperative manner. (A) Possible models of *S. pombe* septin complex organization. (B–E) anti-myc (left side of panels) and anti-GFP or anti-HA (right side of panels) immunoprecipitates from the indicated strains were blotted with anti-myc (top panels) and anti-GFP or anti-HA (bottom panels) antibodies.

conclude that *S. pombe* septin complexes contain at least two copies of each subunit. Considering the sedimentation value of 8.5S, the proteomics data, and the similarity with the *S. cerevisiae* septin complex (Frazier *et al.*, 1998), it is probable that each *S. pombe* septin complex contains just two copies of each subunit, although this remains to be proven rigorously.

S. pombe *Septins Associate in a Cooperative Manner*

Thus far, our data supported a model in which two copies of Spn3p-Spn4p-Spn1p-Spn2p are arrayed in an almost linear order in which each *S. pombe* septin directly contacts its identical partner as well as one or two nonidentical subunits (Figure 4A, left panel). To determine whether there were cooperative interactions between septins to facilitate homotypic interactions, we examined which septins maintained homotypic interactions in the absence of other components. Again, diploid strains producing two different epitope-tagged alleles of each septin in all combinations of deletion strains were used in immunoprecipitation experiments.

First, the role of Spn1p, Spn3p, and Spn4p in the Spn2p-Spn2p interaction was examined. In the absence of *spn1*, *spn3*, or *spn4*, anti-HA immunoprecipitates from the double-tagged strains but not the single-tagged strains contained Spn2p-myc in addition to Spn2p-HA (Figure 4B). Conversely, anti-myc immunoprecipitates from the double-tagged strains but not the single-tagged strains contained Spn2p-HA in addition to Spn2p-myc (Figure 4B). Therefore,

we conclude that the Spn2p-Spn2p interaction is independent of the other three septins.

Next, we examined the Spn1p-Spn1p interaction in the absence of *spn2*, *spn3*, or *spn4*. In lysates prepared from *spn1-myc spn2::ura4/spn1-CFP spn2::ura4*, *spn1-myc spn3::ura4/spn1-CFP spn3::ura4*, or *spn1-myc spn4::ura4/spn1-CFP spn4::ura4* diploid cells, antibodies to GFP precipitated both Spn1p-CFP and Spn1p-myc, and conversely anti-myc immunoprecipitations contained both Spn1p-myc and Spn1p-CFP. Coprecipitating proteins that interacted specifically with the antibodies were not observed in strains producing single epitope–tagged proteins (Figure 4C). Therefore, the Spn1p-Spn1p interaction, like the Spn2p-Spn2p interaction, is independent of other septins.

In contrast, we found that the ability of Spn3p and Spn4p to self-associate depended on certain of the other septins. In the absence of *spn1*, anti-HA immunoprecipitates from the *spn4-myc spn1::ura4/spn4-HA spn1::ura4* double-tagged strain did not contain Spn4p-myc; the only protein detected in either the single- or double-tagged strains was Spn4p-HA (Figure 4D). Reciprocally, anti-myc immunoprecipitates from the *spn4-myc spn1::ura4/spn4-HA spn1::ura4* double-tagged strain did not contain Spn4-HA either; the only protein detected in either the single- or double-tagged strains was Spn4p-myc (Figure 4D). However, in lysates prepared from *spn4-mycspn2::ura4/spn4-YFPspn2::ura4* or *spn4-myc spn3::ura4/spn4-HA spn3::ura4* diploid cells, antibodies to GFP or HA did precipitate Spn4p-myc in addition to either Spn4p-YFP or Spn4p-HA, and conversely anti-myc immunoprecipitations contained both Spn4p-YFP or Spn4p-HA in addition to Spn4p-myc. Therefore, the Spn4p-Spn4p interaction did require Spn1p but did not require Spn2p or Spn3p.

Lastly, the Spn3p-Spn3p interaction was found to be independent of *spn2* but dependent on *spn1* and *spn4* (Figure 4E). Anti-HA immunoprecipitates from the *spn3-myc spn1::ura4/spn3-HA spn1::ura4* or *spn3-myc spn4::ura4/spn3-HA spn4::ura* double-tagged strain did not pull down Spn3p-myc; the only protein detected in either the single- or double-tagged strains was Spn3p-HA (Figure 4E). Reciprocally, anti-myc immunoprecipitates from the *spn3-myc spn1::ura4/spn3-HA spn1::ura4* or *spn3-myc spn4::ura4/spn3-HA spn4::ura* double-tagged strain did not pull down Spn3-HA; the only protein detected in either the single- or double-tagged strains was Spn3p-myc (Figure 4E). In contrast, anti-HA immunoprecipitates from the *spn3-myc spn2::ura4/spn3-HA spn2::ura4* double-tagged strain but not the single-tagged strain contained Spn3p-myc in addition to Spn3p-HA (Figure 4E). Conversely, anti-myc immunoprecipitates from the *spn3-myc spn2::ura4/spn3-HA spn2::ura4* double-tagged strain but not the single-tagged strain contained Spn3p-HA in addition to Spn3p-myc (Figure 4E).

In summary, the model consistent with all of our biochemical data is illustrated in Figure 4A, right panel, which indicates a branch in the complex because we have gathered no evidence that Spn3p and Spn4p are able to directly associate with themselves. However, the reduced recoveries of coprecipitating proteins in some cases (Figure 4B, top panel, and D, middle panel) and the absence of coprecipitating proteins in others indicate that most heterotypic septin interactions within the complex have either stabilizing effects or are absolutely required for homotypic septin interactions. Considering this and the straight edges of septin filaments observed by electron microscopy (Field *et al.*, 1996; Frazier *et al.*, 1998; Kinoshita *et al.*, 2002; Mendoza *et al.*, 2002; Versele and Thorner, 2004), we must also consider the model illustrated in Figure 4A, left panel, for septin organization and ultrastructural analysis will be required to distinguish between the two.

Interdependence of Septins for Ring Formation

Spn3p-GFP and Spn1p-GFP/CFP were shown previously to be localized diffusely during interphase and then organized into a ring structure at the end of anaphase (Berlin *et al.*, 2003; Tasto *et al.*, 2003). This ring splits as the septum forms but does not constrict with actomyosin ring invagination; rather, the ring dissipates after cell cleavage (Berlin *et al.*, 2003; Tasto *et al.*, 2003). Spn4p-YFP and Spn2p-GFP localized identically to Spn1p and Spn3p (Figure 5, A and B). To determine which septin molecules or assemblies of molecules were critical for this localization pattern, we used GFP/CFP/YFP-tagged strains in combination with deletion alleles.

In the absence of Spn1p, the remaining septins showed no specific localization (Figure 5, C, E, and G). Likewise, Spn1p, Spn2p, and Spn3p failed to concentrate at the medial cortex in *spn4*Δ cells (Figure 5, D, F, and H). When these observations are considered in light of the model arising from our biochemical data (Figure 4A), we conclude that neither Spn3p or Spn2p alone, nor the subcomplexes of Spn3p-Spn4p or Spn2p-Spn1p can be recruited to the medial region of cells or form ring structures. It is not surprising then that the cell separation defect of *spn1*Δ and *spn4*Δ cells is as severe as the loss of all *spns* (unpublished data).

In *spn2*Δ cells, we found that Spn1p, Spn4p, and Spn3p localized to ring structures (Figure 6, A, D, and E). We infer that these rings are functional because most *spn2*Δ cells undergo normal cell separation (Figure 6, A, D, and E) in contrast to *spn1*Δ or *spn4*Δ cells (see Figure 5, C–H). Interestingly, Spn1p, Spn4p, and Spn3p were also detected in ectopic structures in the cytoplasm of *spn2*Δ cells (Figure 6, A, D, and E). To determine whether these structures contained more than one septin, we constructed *spn2*Δ strains expressing Spn1p-CFP and Spn4p-YFP or, Spn1p-CFP and Spn3p-GFP. In both strains, the septins colocalized in rings and ectopic structures (Figure 6, D and E), results consistent with the biochemical analysis of septin complexes in *spn2*Δ cells presented above that indicated Spn1p, Spn3p, and Spn4p remained together in the absence of Spn2p. Similarly, Spn1p-CFP, Spn2p-GFP, and Spn4p-GFP localized to rings and ectopic structures in *spn3*Δ cells and *spn3*Δ cells also displayed a milder cell separation phenotype (Figure 6, B, C, F, and G) than *spn1*Δ or *spn4*Δ cells (see Figure 5, C–H). Because we used mild fixation for one of our colocalization experiments (Figure 6D), we compared live and fixed cells directly (Figure 6, F and G, and unpublished data) and found no significant difference in septin localization patterns. Septin localization patterns appear to resist ethanol fixation very well. Lastly, we examined the localizations of Spn1p-CFP or Spn4p-YFP in cells lacking both Spn2p and Spn3p. In these strains, Spn1p-CFP and Spn4p-YFP did not form rings (Figure 6F and unpublished data). Consistent with this, the phenotype of *spn2*Δ *spn3*Δ cells is as severe as *spn1*Δ or *spn4*Δ cells (Figure 6F and unpublished data). Most cells contained ectopic septin structures and in some cells, Spn1p-CFP and Spn4p-YFP formed a ring of medially placed "dots" (Figure 6F and unpublished data). Combining this observation with our biochemical data, we conclude that a subcomplex of Spn1p-Spn4p is sufficient for formation of ectopic structures and localizing to the medial cortex, but at least one other septin is required for assembly of a ring structure.

S. pombe *Septin Ring Formation Requires Two Steps*

The results presented above suggest that *S. pombe* septins 1-4 are present in 8.5S complexes during all stages of the cell

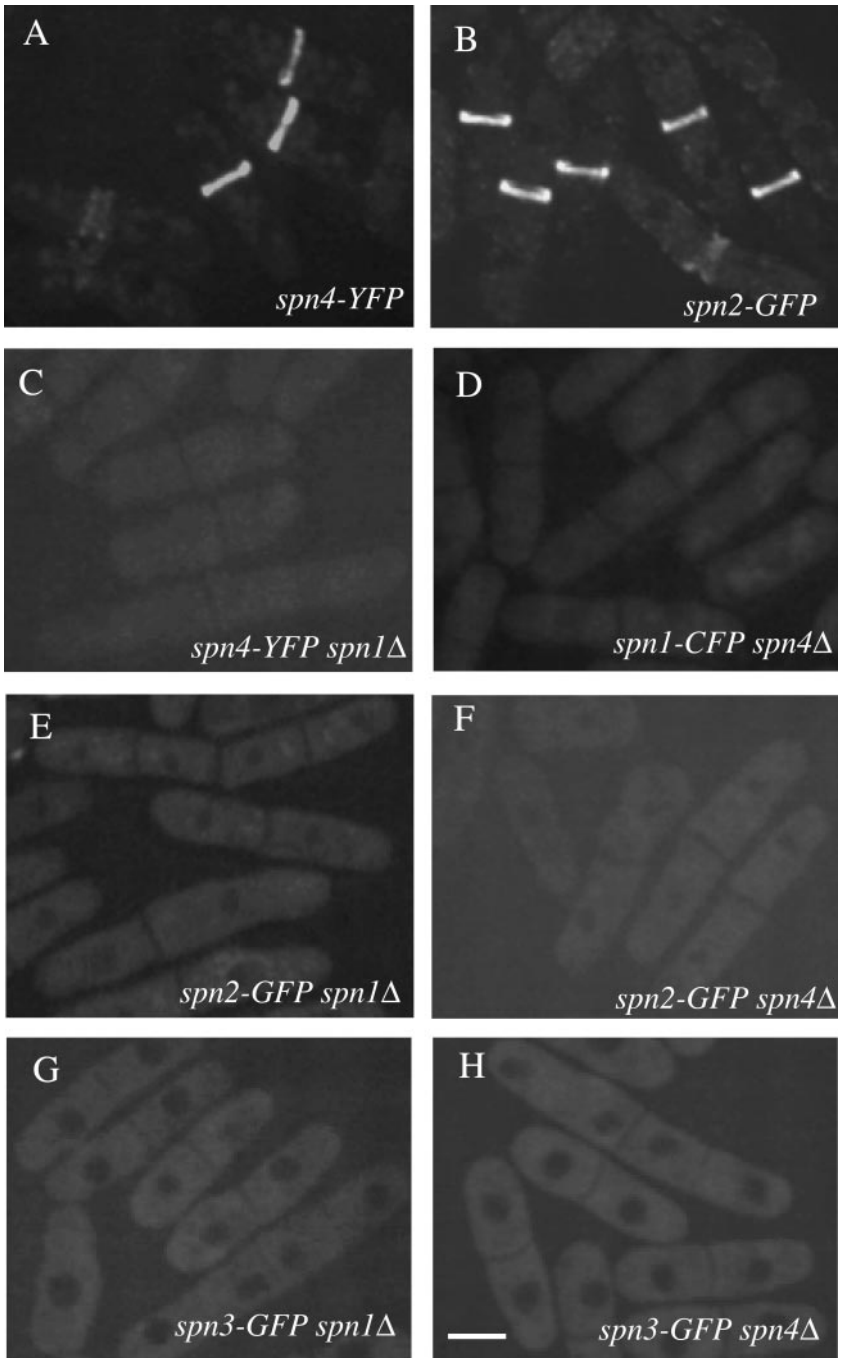

Figure 5. Septin rings are absent in *spn1*Δ and *spn4*Δ cells. The (A) *spn4-YFP* (KGY496), (B) *spn2-GFP* (KGY4230), (C) *spn4-YFP spn1*Δ (KGY842), (D) *spn1-CFP spn4*Δ (KGY4417), (E) *spn2-GFP spn1*Δ (KGY4258), (F) *spn2-GFP spn4*Δ (KGY4259), (G) *spn3-GFP spn1*Δ (KGY4260), and (H) *spn3-GFP spn4*Δ (KGY2281) strains were grown in YE medium at 25°C, and GFP-tagged proteins were visualized in live cells. Scale bar, 5 μm.

cycle except late mitosis when they are recruited to the medial cortex and organize into higher order oligomers that are visualized as rings. To determine whether septin organization into higher order structures requires entry into mitosis, we examined the ability of synchronized cultures of *cdc16-116* cells to form septin rings when shifted to their nonpermissive temperature. Cdc16p is a component of the bipartite GAP for the small GTPase, Spg1p, whose activation triggers the septation initiation network (SIN; Schmidt *et al.*, 1997; Furge *et al.*, 1998). The SIN induces actin ring constriction and septum formation normally as cells exit mitosis (McCollum and Gould, 2001). When Cdc16p function is abrogated during interphase, septation can occur before mitosis (Minet *et al.*, 1979), and this allowed us to ask whether septin rings could form during interphase. *cdc16-116 spn3-GFP* cells synchronized in early G2 phase were obtained by centrifugal elutriation and shifted to 36°C. Samples were collected periodically to examine nuclear division, septum formation, and formation of Spn3p-GFP rings (Figure 7A). At 60 min after shift to 36°C, Spn3p-GFP had accumulated in the medial region of cells and septa began to form in a population of interphase cells. However, close examination of these cells revealed that Spn3p-GFP staining was more diffuse than in wild-type cells and extended across the entire septum in a disk rather than being restricted to the cortex (Figure 7C). It was never visualized as a split ring in these cells (Figure 7C). In contrast, Spn3p-GFP rings were tight, focused, and cortically restricted in cells that had passed into

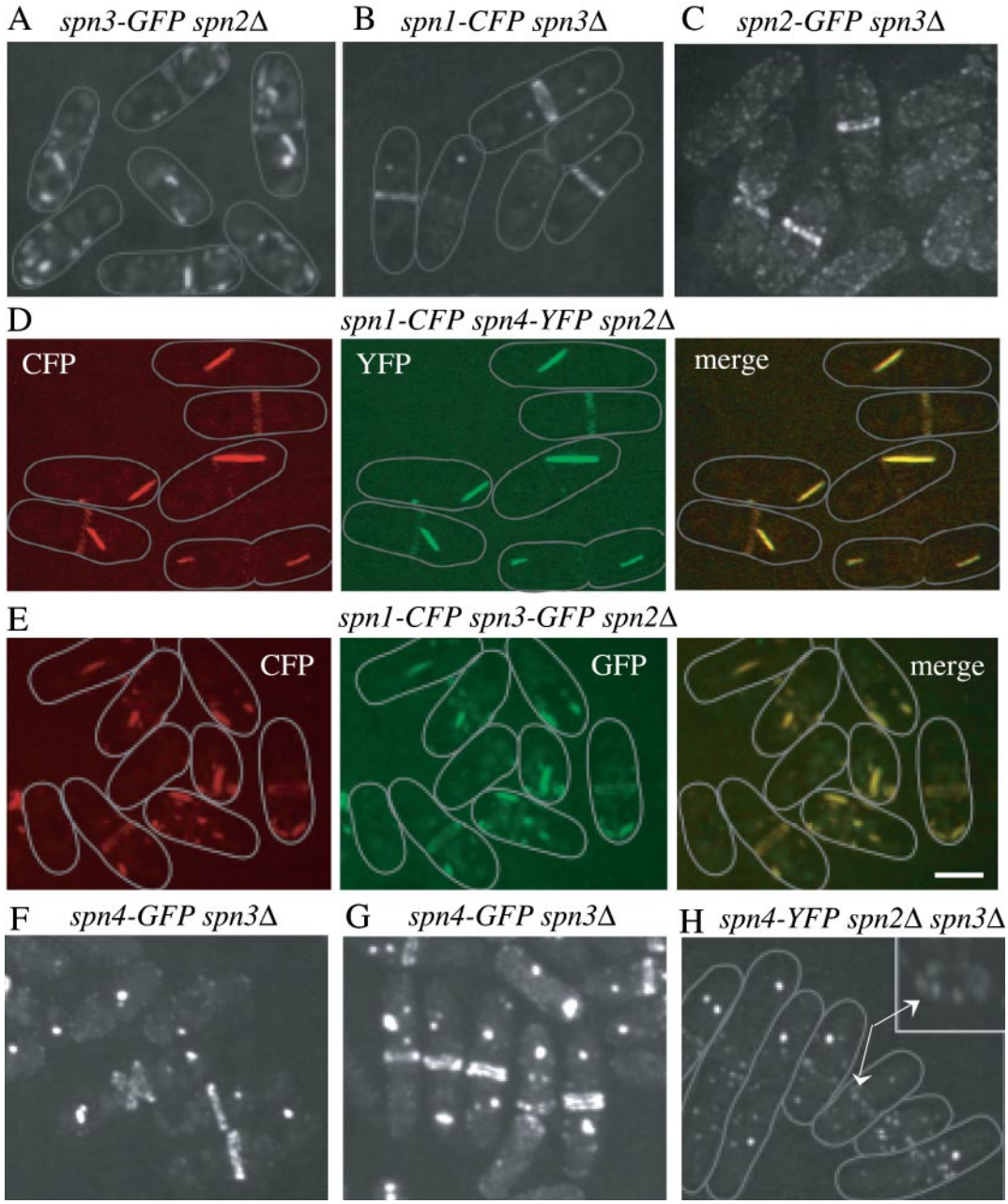

Figure 6. Interdependence of septins for ring formation. (A) *spn3-GFP spn2*Δ (KGY3211), (B) *spn1-CFP spn3*Δ (KGY730), or (C) *spn2-GFP spn3*Δ (KGY3169) cells were grown at 25°C, and GFP-tagged proteins were visualized in live cells. (D) *spn1-CFP spn4-YFP spn2*Δ (KGY761) cells were fixed in ethanol and both Spn1p-CFP and Spn4p-YFP were photographed separately, and then the images merged. (E) *spn1-CFP spn3-GFP spn2*Δ (KGY887) cells were grown at 25°C, and Spn1p-CFP and Spn3p-GFP were photographed separately from live cells, and the images were merged. (F and G) *spn4-GFP spn3*Δ cells (KGY5000) were grown at 25°C, and either visualized live in (F) or fixed in ethanol before visualization (G). (H) Spn4p-YFP was visualized in live *spn4-YFP spn2*Δ *spn3*Δ cells (KGY1178). The inset shows the middle cell rotated on its *Z*-axis. Arrows point to the arrangement of Spn4p-YFP "dots" in the medial region of the cell. Gray lines represent the outlines of individual cells. Scale bar, 5 μm.

and through mitosis before septum formation; in these cells split rings were easily detected (Figure 7D). We also examined the recruitment of Mid2p to the medial cortex in *cdc16-116* cells at restrictive temperature. Consistent with the cell cycle regulation of Mid2p abundance and localization observed previously (Tasto *et al.*, 2003), Mid2p rings were detected only in cells that had passed through mitosis before septum formation (Figure 7, B and E). Further, the appearance of Mid2p at the cortex coincided with the assembly of organized septin rings (compare 150 min time points; Figure 7, D and E). These findings indicate that ring formation and septation can drive recruitment of septin complexes to the medial region of the cell but another factor(s) available only in mitosis, such as Mid2p, alone or in combination with other factors is required for proper septin ring organization.

To determine whether Mid2p is the sole additional factor required for septin ring organization in interphase *cdc16-116* cells, we mildly overproduced HA-Mid2p from the *nmt41*

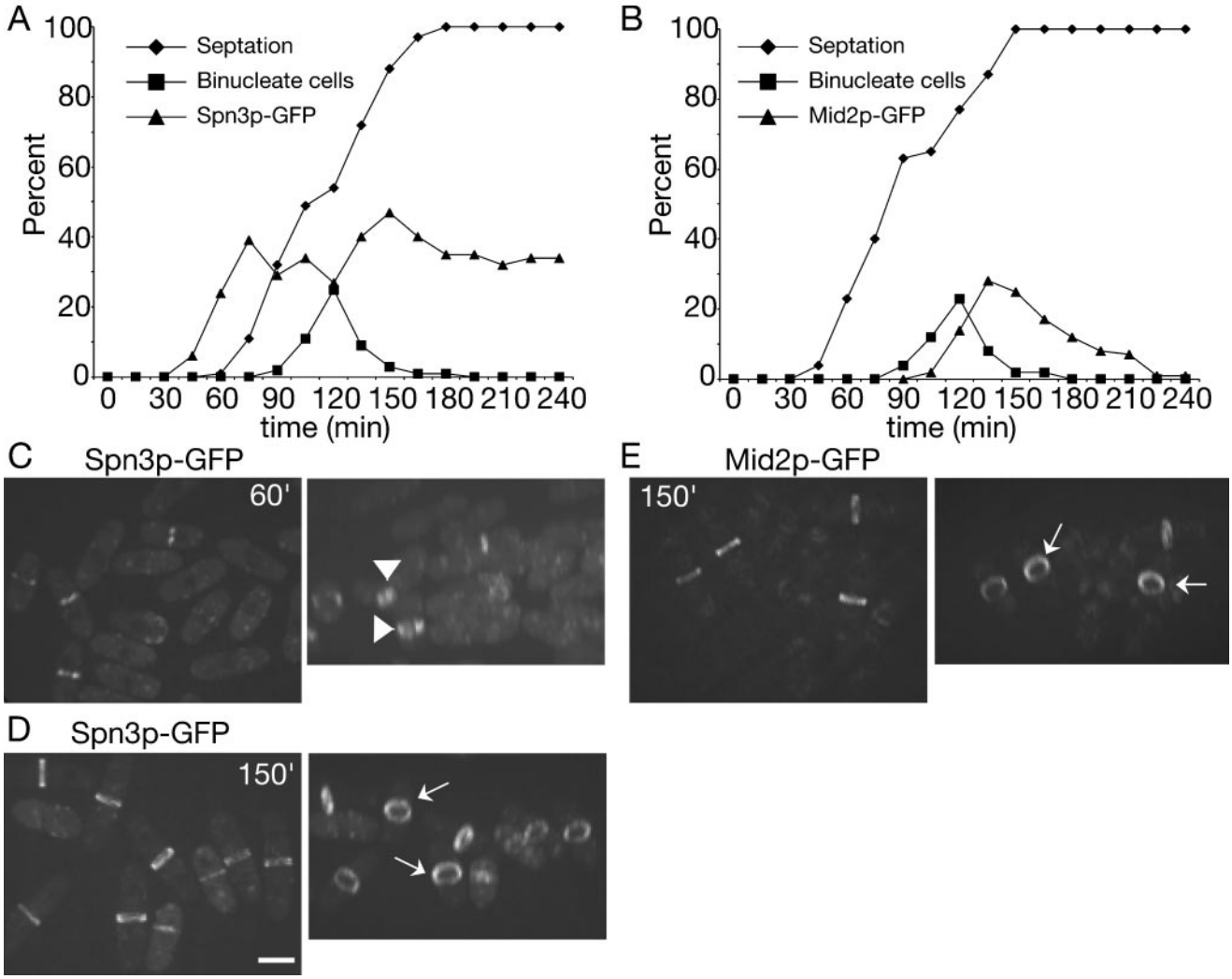

Figure 7. Septin recruitment to the cortex, but not stable ring formation, can be induced during interphase. (A) *spn3-GFP cdc16-116* (KGY738) and (B) *mid2-GFP cdc16-116* (KGY741) cells were grown to midlog phase at 25°C, synchronized in early G2 phase by centrifugal elutriation, and then shifted to 36°C. Samples of cells were collected every 15 min. One portion of each sample was imaged immediately to determine the percentage of septated cells and the percentage of cells with Spn3p-GFP and Mid2p-GFP medial localization, respectively. Another portion was fixed with ethanol immediately and then stained with DAPI to determine the percentage of binucleate cells. (C and D) Spn3p-GFP localization in live cells from (C) 60 min or (D) 150 min time points (left panels of each). Images in the left panels were also rotated in the *Z*-axis to better reveal septin ring organization (right panels). Short arrows point to disorganized septin rings at 60 min. Longer arrows point to organized rings at 150 min. (E) Mid2p-GFP localization in live cells from 150 min time point. Cells are also rotated in the *Z*-axis to better show Mid2p ring organization. Arrows point to organized Mid2p rings. Scale bar, 5 μm.

promoter before selecting G2 phase cells by centrifugal elutriation. In this case, Spn3p-GFP was recruited to the medial region before mitosis as before (Figure 8A) and it was now assembled into highly organized ring structures that were easily visualized as split rings once septa had formed (Figure 8, B and C). Virtually no diffuse disk structures were observed (Figure 8, B–D). We conclude from this experiment that Mid2p is solely responsible directly or indirectly for regulating septin ring coalesence in *S. pombe*. Another noteworthy consequence of mild Mid2p overexpression was the relative persistence of septin rings and the inhibition of mitotic progression, as determined by monitoring the formation of binucleates (Figure 8A). This result is consistent with our previous results, indicating that prolonged expression of Mid2p stabilizes septin ring structures and influences cell cycle progression (Tasto *et al.*, 2003).

DISCUSSION

In this study, we have determined the basic organization of *S. pombe* septin complexes that are used to build septin rings during cytokinesis and defined two discrete steps in septin ring assembly. Because the four septins that participate in *S. pombe* cell division are not essential for viability, we were able to assess the interdependence and function of each septin in the absence of any one or combination of the others. The model of septin complex organization arising from these studies indicates that Spn1-4p form an almost linear array of a specific order that is held together by a series of interdependent associations (Figure 4A). Further, different subunits within this complex are not equivalent in their functions. Our results have implications for understanding the nature of septin complexes and their properties in other eukaryotes.

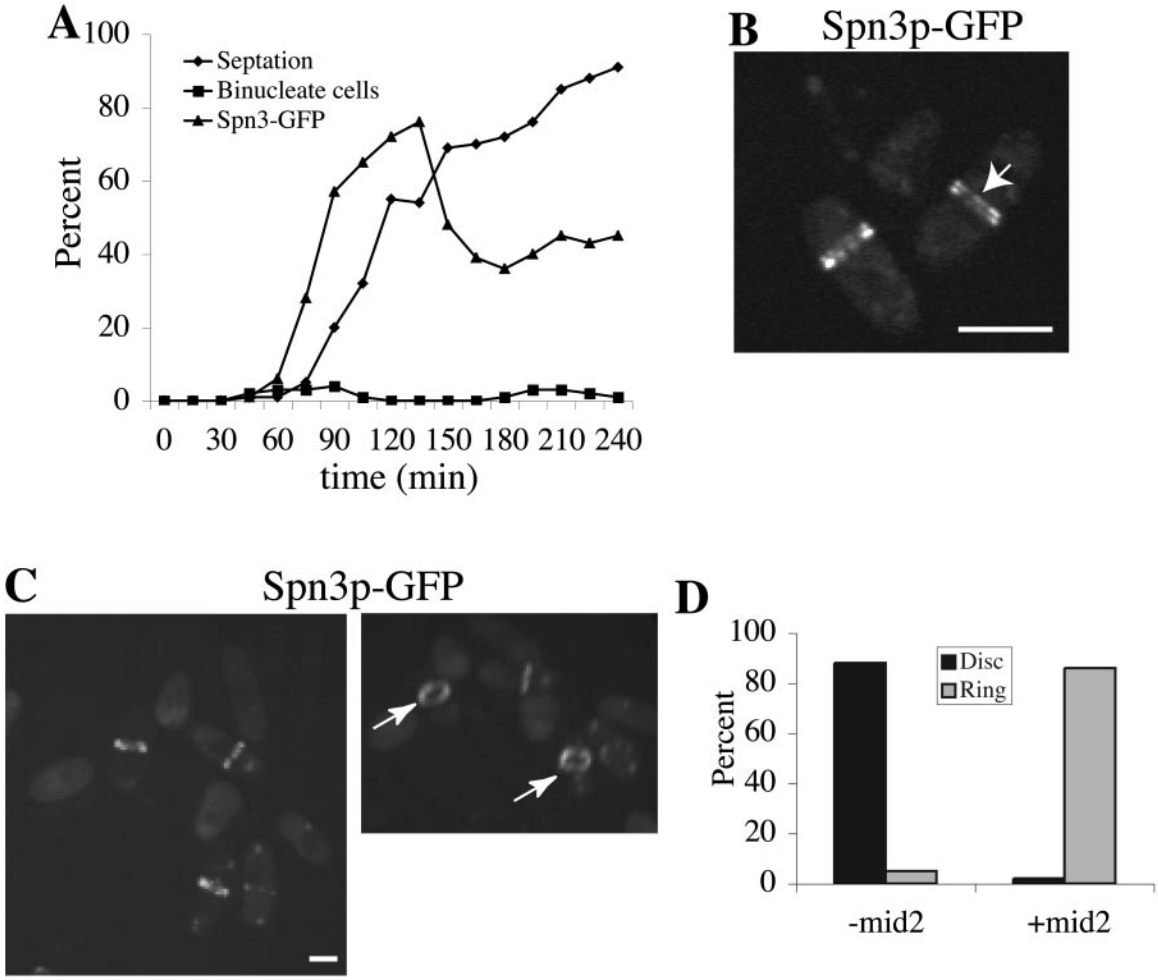

Figure 8. Coalescence of septin rings can be induced during interphase by *mid2*$^+$ expression. (A) *spn3-GFP cdc16-116* (KGY738) cells were transformed with pREP41-*HA-mid2*$^+$. Transformants obtained in the presence of thiamine in the medium were then grown to midlog phase in the absence of thiamine at 25°C for 15 h. They were then synchronized in early G2 phase by centrifugal elutriation and immediately shifted to 36°C. Samples of cells were collected every 15 min. One portion of each sample was imaged immediately to determine the percentage of septated cells and the percentage of cells with Spn3p-GFP medial localization. Another portion was fixed with ethanol and stained with DAPI to determine the percentage of binucleate cells. (B and C) Spn3p-GFP localization in live cells at the 75-min time point. In B, cells imaged with a single *Z*-series stack (0.49 μm) are shown to provide the best visualization of split septin rings as indicated by the arrow. In C, the image in the left panel represents a 3D reconstruction of *Z*-series stacks. This image was rotated in the *Z*-axis (right panel) to illustrate the cortical restriction of the septin rings. The arrows point to organized septin rings. (C) Quantitation of disorganized septin rings (disk) and organized septin rings (ring) in interphase *cdc16-116* cells in the presence or absence of Mid2p. Percentages were determined by visual inspection of *Z*-series stacks of live cells that contained medially placed septin structures from the experiment described in Figure 7A, 75-min time point, 93 cells examined (−mid2) and from the experiment described in part A of this figure, 75-min time point, 88 cells examined (+mid2). Scale bar, 5 μm.

First, our data argue that each septin does not possess the intrinsic ability to localize to its site of action or to form ring structures, a possibility raised by the finding that an individual septin subunit (*Xenopus laevis* Sept2) can polymerize into filaments in vitro (Mendoza *et al.*, 2002). Not only do our findings indicate that individual septin subunits do not normally exist on their own in yeast (in *S. pombe*, the minimal unit comprises 8 polypeptides), our data also indicate that ring formation in *S. pombe* requires a minimum of three complexed septins, either Spn3p-Spn4p-Spn1p or Spn4p-Spn1p-Spn2p. That the complexes of three septins are sufficient to form ring structures explains why the absence of *spn2*$^+$ or *spn3*$^+$ generates a mild cell separation defect relative to the deletion of *spn4*$^+$ or *spn1*$^+$ or both *spn2*$^+$ and *spn3*$^+$.

Interestingly, our biochemical and cell biological analyses of various septin deletion strains indicate that the Spn4p-Spn1p subcomplex but not the Spn3p-Spn4p or Spn1p-Spn2p subcomplexes is able to accumulate as dot structures in the medial region of cells. Although a conserved polybasic region within septins binds phosphatidyl inositol biphosphate in vitro and has been implicated in septin localization (Zhang *et al.*, 1999; Casamayor and Snyder, 2003), our results suggest that the Spn1p-Spn4p subcomplex contains the targeting information for the complex and it will be interesting in the future to illuminate its nature. We also observed that subcomplexes of two (Spn4p-Spn1p) or three (Spn3p-Spn4p-Spn1p or Spn4p-Spn1p-Spn2p) septins formed ectopic structures that were not observed in wild-type cells expressing the full complement of the four cytokinetic septins. This

raises the possibility that subunits on the end of the array (Spn2p or Spn3p) might normally act in interphase to prevent spurious assembly into aggregates or higher order structures. Purified septins have been observed to polymerize into filaments in vitro (Field *et al.*, 1996; Frazier *et al.*, 1998; Kinoshita *et al.*, 2002; Mendoza *et al.*, 2002). If the ectopic structures observed in vivo are due to septin filament formation rather than aggregation, our observations may be relevant to the findings that individual septins and complexes of septins display differential abilities to polymerize in vitro (reviewed in Mitchison and Field, 2002). Because ectopic structures were only observed with the particular subcomplexes described above, the outcome of in vitro studies might depend on which septin or septin subcomplex is chosen for examination. On that note, our model of septin organization and function suggests that the integrity of the complex as a whole should be examined as part of any structure/function analysis performed on individual subunits. For example, any mutation of either Spn2p (Cdc10p) or Spn3p (Cdc11p) that disturbs its interaction with Spn1p (Cdc3p) or Spn4p (Cdc12p), respectively, would be expected to negatively influence its ability to localize appropriately but not necessarily because an intrinsic targeting domain was disturbed.

Several observations suggest that the organization of septins we have found in *S. pombe* will be relevant to other septin complexes, at least to those found in *S. cerevisiae*. First, *S. cerevisiae* cells lacking Cdc10p or Cdc11p (Spn2p and Spn3p homologues, respectively) contain complexes of the other three septins (Frazier *et al.*, 1998). Second, such complexes retained some function because the cells divided albeit with reduced efficiency (Frazier *et al.*, 1998), a situation similar to what is observed in *spn2*Δ and *spn3*Δ cells. Third, subsets of *S. cerevisiae* proteins produced in *E. coli* comparable to those we have found associate with one another in *S. pombe* were able to complex with one another and form filamentous structures (Versele *et al.*, 2004; Versele and Thorner, 2004). Lastly, two-hybrid results testing *S. cerevisiae* septin interactions support our working models (Figure 4A), although they should be interpreted with caution because of the strong possibility of bridging interactions. These analyses suggest that Cdc11p (Spn3p homolog) interacts with Cdc12p (Spn4p homolog; Lee *et al.*, 2002; Casamayor and Snyder, 2003), Cdc12p interacts strongly with Cdc3p (Spn1p homolog) (Lee *et al.*, 2002), and Cdc3p interacts with Cdc10p (Spn2p homolog; Lee *et al.*, 2002), and there is an absence of strong interaction between Cdc3p and Cdc11p (Lee *et al.*, 2002; Casamayor and Snyder, 2003).

Precocious activation of the septation initiation network (SIN) induced septins to concentrate at the position of the actomyosin ring in interphase cells. However these septin structures were not organized properly. Indeed, they were very reminiscent of septin structures formed in cells lacking Mid2p (Berlin *et al.*, 2003; Tasto *et al.*, 2003) and in fact Mid2p-GFP was not visualized at rings induced by activation of the SIN during interphase. Thus, we conclude that septin accumulation at the medial region of the cell and coalescence into a ring structure are genetically separable events. The first requires an event(s) that can be initiated by actomyosin ring formation and SIN activity but is independent of mitotic entry. By supplying Mid2p artificially to interphase *cdc16-116* cells, coalescence into ring structures was induced. This result indicates that Mid2p, directly or indirectly, is solely responsible for septin ring coalescence. The two-step assembly of *S. pombe* septin rings described here may be analogous to the situation in *S. cerevisiae* in which septin rings in unbudded cells are relatively unstable and become stabilized as cells proceed to bud (Caviston *et al.*, 2003; Dobbelaere *et al.*, 2003). Mid2p has a homolog in *S. cerevisiae*, Bud4p (Sanders and Herskowitz, 1996), which is similarly cell cycle regulated, but it is not known whether Bud4p participates in septin ring organization.

An established regulatory step for *S. cerevisiae* septin ring formation is septin phosphorylation, and several protein kinases have been implicated in septin ring dynamics including the GIN4 family, Cdk1p, and Cla4p (Cvrckova *et al.*, 1995; Longtine *et al.*, 1998; Weiss *et al.*, 2000; Mortensen *et al.*, 2002; Tang and Reed, 2002; Dobbelaere *et al.*, 2003; Schmidt *et al.*, 2003; Versele and Thorner, 2004). Although the *S. pombe* homologues of the GIN4 kinases, Cdr1p and Cdr2p, do not influence septin ring organization (Morrell *et al.*, 2004), it will be interesting to determine whether *S. pombe* septin ring assembly is regulated by phosphorylation and whether Mid2p acts in concert with protein kinases to control this process. Another factor implicated in the organization of *S. cerevisiae* septin rings is a fifth septin, Shs1p/Sep7 (Mortensen *et al.*, 2002; Dobbelaere *et al.*, 2003). *S. pombe* does not have an ortholog of Shs1p/Sep7 and we did not detect a fifth septin copurifying with Spn1-4p-TAP complexes. The *S. pombe* genome does encode an additional three septins but their expression is restricted to meiosis (Mata *et al.*, 2002; Rustici *et al.*, 2004). Thus, it is unclear whether the role of Shs1p/Sep7 in *S. cerevisiae* septin ring organization will extend to other organisms.

As both recombinant and purified septins can polymerize into filaments in vitro (Field *et al.*, 1996; Frazier *et al.*, 1998; Kinoshita *et al.*, 2002; Mendoza *et al.*, 2002; Versele and Thorner, 2004), it is interesting to consider how the complex described here is assembled into such higher order oligomers. It will be informative in future studies to link the ability of particular septin complexes to form filamentous structures in vitro with the in vivo requirements of ring formation we have established here.

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