Mid2p stabilizes septin rings during cytokinesis in fission yeast

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Septins are filament-forming proteins with a conserved role in cytokinesis. In the fission yeast *Schizosaccharomyces pombe*, septin rings appear to be involved primarily in cell–cell separation, a late stage in cytokinesis. Here, we identified a protein Mid2p on the basis of its sequence similarity to *S. pombe* Mid1p, *Saccharomyces cerevisiae* Bud4p, and *Candida albicans* Int1p. Like septin mutants, *mid2Δ* mutants had delays in cell–cell separation. *mid2Δ* mutants were defective in septin organization but not contractile ring closure or septum formation. In wild-type cells, septins assembled first during mitosis in a single ring and during septation developed into double rings that did not contract. In *mid2Δ* cells, septins initially assembled in a single ring but during septation appeared in the cleavage furrow, forming a washer or disc structure. FRAP studies showed that septins are stable in wild-type cells but exchange 30-fold more rapidly in *mid2Δ* cells. Mid2p colocalized with septins and required septins for its localization. A COOH-terminal pleckstrin homology domain of Mid2p was required for its localization and function. No genetic interactions were found between *mid2* and the related gene *mid1*. Thus, these studies identify a new factor responsible for the proper stability and function of septins during cytokinesis.

Introduction

Septins are a class of GTPase proteins with functions in cytokinesis conserved from yeast to mammalian cells (Longtine et al., 1996; Field and Kellogg, 1999; Kartmann and Roth, 2001). Septin proteins were first identified in budding yeast where they are localized to rings at the bud neck. The ability of septins to form filaments in vitro and their association with filament-like structures at the bud neck in electron micrographs have led to the proposal that they constitute bud neck filaments (Byers and Goetsch, 1976; Longtine et al., 1996, 1998; Frazier et al., 1998). In budding yeast, septins are required for cytokinesis and are thought also to function as a scaffold for the localization of many signaling proteins, cell cycle regulators, bud site selection proteins, and chitin synthases (Chant, 1996; Longtine et al., 1996; Field and Kellogg, 1999; Gladfelter et al., 2001). The identification of septins in other organisms revealed that their role in cytokinesis is conserved. For example, a *Drosophila* septin, Pnut, is localized to the cleavage furrow and is required for cytokinesis of certain cell types (Neufeld and Rubin, 1994; Adam et al., 2000). Recent studies on many other septins have uncovered a diversity of functions, including sporulation, association with secretory proteins, and association with stress fibers in nondividing cells (Kartmann and Roth, 2001). Although a number of septin-interacting proteins have been identified, most of these are thought to use septins as a scaffold for localization and proper function. Still little is known about what proteins may help assemble and organize the septins themselves in the cell.

In the fission yeast *Schizosaccharomyces pombe*, cytokinesis proceeds in multiple phases: in early mitosis (preanaphase), a single contractile ring, consisting of actin, myosin, and other proteins, is assembled and persists through anaphase (∼20–30 min). At the end of anaphase, septation is triggered by the Sin/Sid pathway of cell cycle regulators (McCollum and Gould, 2001). During this process, the contractile ring begins to close, acting to guide the closure of the plasma membrane behind it. At the same time, the cell wall of the septum is synthesized outside the plasma membrane. Upon completion of the cell wall and after the cell membranes are closed, cell–cell separation occurs by the digestion of the primary septum. In *S. pombe*, seven septins have been identified, four of which are localized to the division plane and three of which are involved in sporulation in meiotic cells (J. Pringle, personal communication). *S. pombe* septin (*spn*) mutants are viable but have a defect in cell–cell separation and accumulate in

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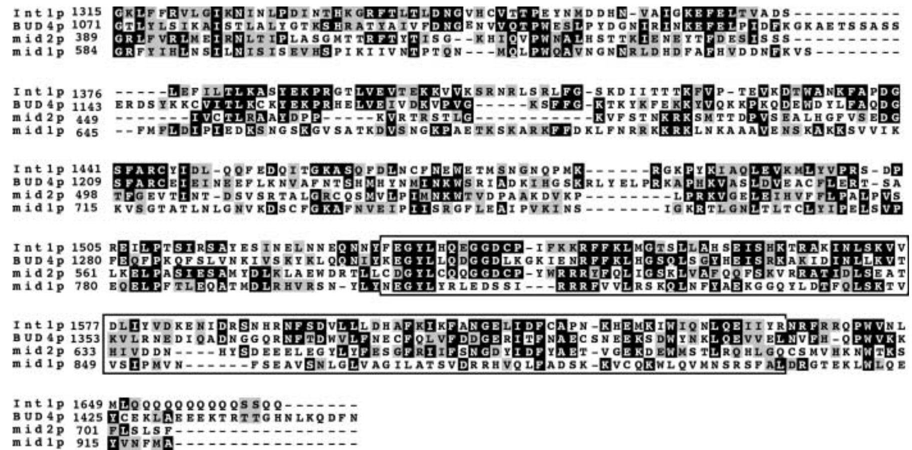

Figure 1. **Mid2p is similar to Mid1p, Bud4p, and Int1p.** Multiprotein alignment of the homologous COOH-terminal regions. Boxed region indicates the PH domains.

chains of cells (Longtine et al., 1996) (J. Pringle, personal communication). Consistent with this phenotype, septins appear at the division site only after the contractile ring has been fully assembled. Thus, in contrast to septins in budding yeast, fission yeast septins are not essential for cell viability and may function primarily in late stages of cytokinesis.

Here, we identify a gene that functions in septin organization. Mid2p was identified on the basis of its homology to Mid1p, a protein required for proper positioning of the contractile ring during cytokinesis (Sohrmann et al., 1996; Bahler et al., 1998a; Paoletti and Chang, 2000). The Mid2p sequence also shares similarity with Bud4p, a septin-associated protein required for bud site selection in *S. cerevisiae* (Sanders and Herskowitz, 1996), and *Candida albicans* Int1, a protein required for hyphal growth, adhesion, and pathogenesis (Gale et al., 1996, 1998). Our analysis showed that Mid2p associates with and organizes the septin rings. Time-lapse and FRAP analysis of *mid2Δ* mutant cells revealed that Mid2p has a specific role in maintaining the integrity and stability of the septin rings during cleavage.

Results

mid2Δ mutants have a defect in cell–cell separation

Mid2p was identified in a BLAST search of the *S. pombe* genome databases (Sanger Centre, Cambridge, UK) on the basis of its significant amino acid similarity to *S. pombe* Mid1p (19% identical residues, 37% similar, expect value $2e^{-05}$) (Altschul et al., 1990; Sohrmann et al., 1996; Tatusova and Madden, 1999). Mid2p also possess significant homology to *S. cerevisiae* Bud4p (20% identical, 38% similar, expect value $7e^{-13}$), and *C. albicans* Int1p (25% identical, 47% similar in the COOH-terminal 336 aa, expect value $4e^{-24}$) (Gale et al., 1996; Sanders and Herskowitz, 1996). All of these proteins possess a pleckstrin homology (PH)* domain at their very COOH terminus and share similarity in an adjacent region at the COOH terminus (Fig. 1). Mid1p and Bud4p also share homology through the entire length of Mid2p.

mid2Δ deletion strains were generated using a PCR-based homologous recombination gene-targeting system (Bahler et al., 1998b). Heterozygous deletions were initially generated in diploid strains, and sporulation of these diploids yielded viable *mid2Δ* haploid colonies. *mid2Δ* cells grew at wild-type rates both on plates and in liquid cultures, at a range in temperatures (20–36°C), and at high salt (1 M KCl) conditions.

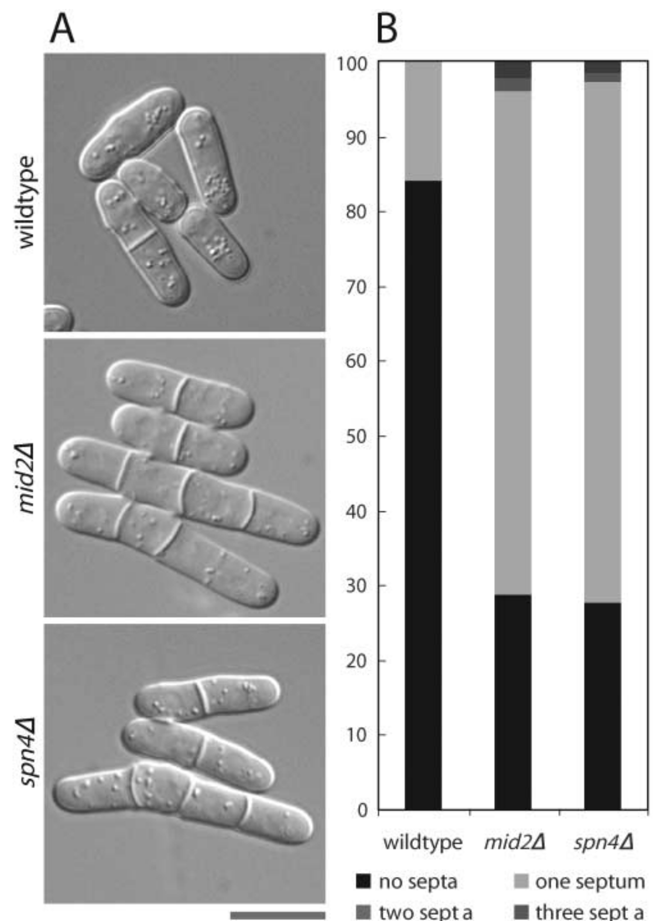

Figure 2. ***mid2Δ* and *spn4Δ* cells have similar cell–cell separation defects.** (A) DIC images of representative wild-type (FC937), *mid2Δ* (FC881), and *spn4Δ* (FC867) mutant cells grown in rich medium at 30°C to the exponential phase of growth. (B) Numbers of septa in wild-type, *mid2Δ*, and *spn4Δ* cells ($n > 400$). Bar, 10 μm.

*Abbreviations used in this paper: DIC, differential interference contrast; PH, pleckstrin homology.

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Microscopic examination of *mid2Δ* cells revealed a significant defect in cell–cell separation in cytokinesis (Fig. 2 A). When grown in rich liquid medium, 16% of asynchronous wild-type cells exhibited a septum, whereas 66% of *mid2Δ* cells possessed one septum (Fig. 2 B). A small percentage (<5% of cells) had two or three septa (grown in rich medium in exponential phase) and grew in short chains of cells. No cells were seen with more than three septa. Careful microscopic examination of the septa in multiple focal planes using differential interference contrast (DIC) or calcofluor staining suggested that most of the septa were complete. Chains of cells occasionally contained a cell that had lysed, suggesting that these mutants had rare defects in cellular integrity and that cells in each chain were completely separated by membrane and septum. Robust growth rates suggest that these mutants do not have a significant delay in progression of the nuclear cell cycle but have a specific delay of approximately one generation time in digestion of the septum for cell–cell separation.

mid2Δ mutant cells exhibit normal contractile rings

Since mild defects in actin-myosin contractile ring organization can lead to cell–cell separation defects (unpublished data), we tested whether Mid2p is involved in the assembly or contraction of the actin ring during cytokinesis. *mid2Δ* cells stained for F-actin with Alexa Fluor phalloidin exhibited well-defined, normal actin rings (unpublished data). Using a Cdc4p (a myosin light chain) fusion to GFP (McCollum et al., 1995; Balasubramanian et al., 1997), we examined if the contraction of the actin-myosin ring might be perturbed. Confocal three-dimensional time-lapse images showed that wild-type Cdc4p-GFP rings contracted at the end of mitosis with a rate of 0.20 ± 0.017 μm/min (n = 5), consistent with previous reports (Bezanilla et al., 2000; Motegi et al., 2000; Pelham and Chang, 2002). In *mid2Δ* cells, Cdc4p-GFP rings appeared normal and contracted at rates similar to those of wild-type rings, 0.17 ± 0.048 μm/min (n = 5; $P > 0.1$) (Fig. 3). Therefore, neither the assembly nor the closure of the contractile ring was markedly perturbed in *mid2Δ* mutants.

mid2Δ mutant cells have defects in organization of septin rings

The phenotype of *mid2Δ* cells was similar to the cell–cell separation phenotype described for *S. pombe* septin mutants (Longtine et al., 1996) (J. Pringle, personal communication). Thus, we compared the *mid2Δ* mutant to a *spn4Δ* (septin 4) mutant. The *spn4Δ* mutant appears to have a septin "null" phenotype, since it has a very similar phenotype to that of cells deleted for all the mitotic septins and exhibits no detectable localization of the other mitotic septins (J. Pringle, personal communication). As expected, *spn4Δ* cells exhibited cell–cell separation defects and accumulated cells with one or more septa (Fig. 2 A). The numbers of septa in *spn4Δ* cells were very similar to those found in *mid2Δ* cells (Fig. 2 B). *spn4Δ mid2Δ* double mutant cells showed a simi-

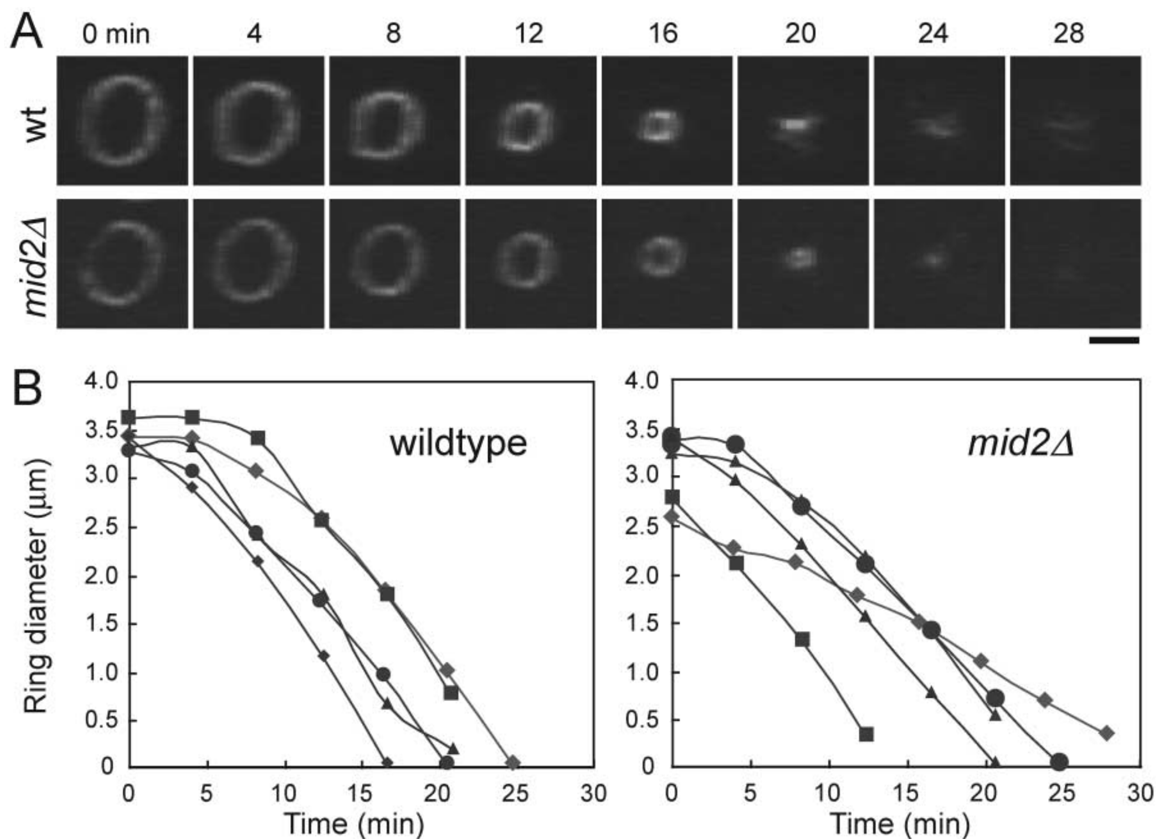

Figure 3. **The contractile ring appears to assemble and close normally in *mid2Δ* cells.** (a) Wild-type and *mid2Δ* cells expressing cdc4-GFP were imaged live using confocal time-lapse microscopy. Z-stacks were rendered in three dimensions to produce a cross-sectional view of the ring. Bar, 2 μm. (B) Measurements of five individual ring diameters over time in wild-type and *mid2Δ* cells.

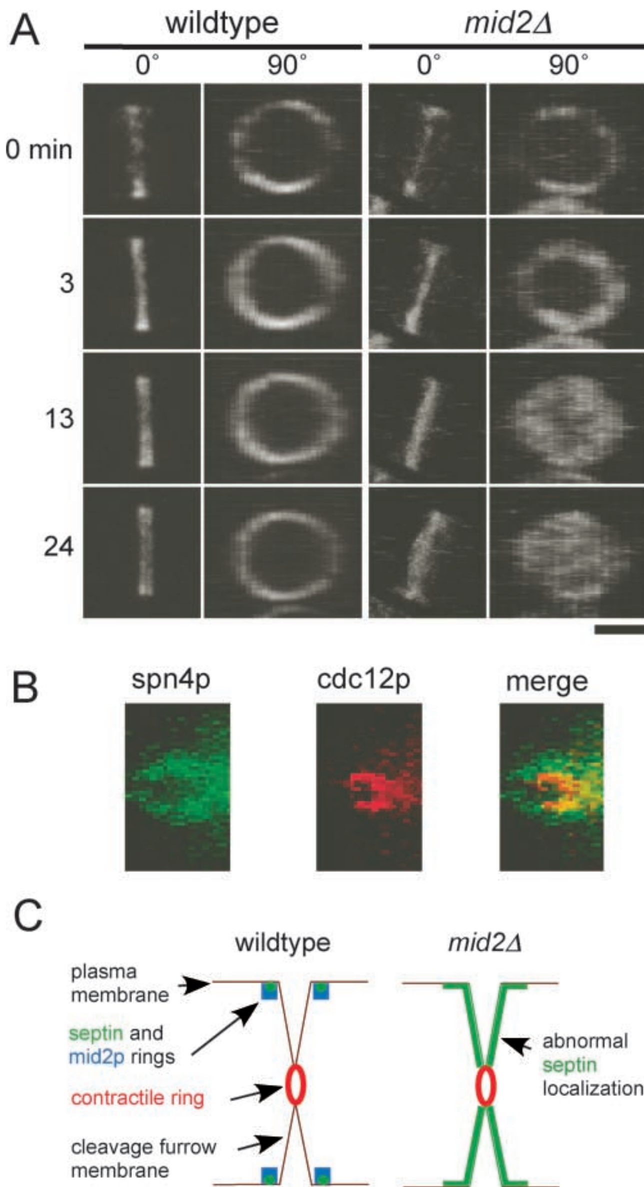

Figure 4. **Organization of septin rings is not maintained in *mid2Δ* cells.** (A) Wild-type (FC937) and *mid2Δ* (FC881) cells expressing Spn4p-GFP were imaged for GFP fluorescence using confocal three-dimensional time-lapse microscopy. Spn4p-GFP structures were rendered in three dimensions at each time point. Side (0°) and cross-sectional (90°) views of the Spn4p-GFP medial structures are shown in wild-type (left) and *mid2Δ* (right) at representative time points. Note that in *mid2Δ* cells, Spn4p-GFP forms a single ring (0 min), an abnormal washer (3–13 min), and then a disc structure (24 min). Bar, 2 μm. (B) *mid2Δ* cells expressing Spn4p-YFP (green) and the contractile ring marker Cdc12-CFP (red) were imaged on a wide field microscope and rendered in three dimensions. Spn4p-YFP is present on the membrane behind the contractile ring in a washer structure. (C) Summary of the distribution of septins in wild-type and *mid2Δ* cells during contractile ring closure.

lar phenotype, suggesting that these proteins act in the same pathway (unpublished data).

Because of this similarity in phenotypes, we tested whether *mid2Δ* cells may have defects in septin organization. We examined septin distribution in living wild-type and *mid2Δ* cells expressing a Spn4p-GFP fusion construct using time-lapse confocal microscopy. In wild-type cells, septins first appeared in anaphase as a collection of medial dots that were then incorporated into a single medial ring around the circumference of the cell (Fig. 4 A). This single ring then changed into a double ring structure that persisted throughout septation. At the end of septation, septins were present in discrete dots at the new cell ends. During interphase, cells sometimes exhibited small septin dots at the cell ends and also occasionally contained a single bright cytoplasmic motile dot or small ring shaped particle (unpublished data).

In *mid2Δ* cells, septin distribution was abnormal. As in wild-type cells, initially Spn4p-GFP localized to a single ring during anaphase (Fig. 4 A). However, at septation the septins did not stay only at the cell perimeter as double rings. Instead, they gradually appeared at the cleavage furrow in the interior of the cell, forming a double washer and then a double disc structure. The rate of invagination in the furrow was 0.16 μm/min, the same rate as the rate of contraction of the contractile ring. Dual labeling of Spn4p-YFP and a contractile ring marker Cdc12p-CFP confirmed that the septins were present in the cleavage furrow behind the actomyosin ring (Fig. 4, B and C). In addition, during septation spn4-GFP was also slightly more spread out on the cell surface (Fig. 4 A). *mid2Δ* cells fixed and stained with anti-Spn4p antibody showed a similar abnormal septin distribution, although the fine structures were not well preserved in fixation (unpublished data). These results suggested that Mid2p has a specific role in maintaining the organization of septin rings during ring contraction.

Mid2p affects septin dynamics

This abnormal localization of Spn4p-GFP in *mid2Δ* cells suggested that septins may be flowing from the septin rings into the cleavage furrow during ring closure. Alternatively, new septin proteins may be deposited at the membrane in the furrow during this process. To distinguish between these two possibilities, we investigated the dynamics of septin proteins using FRAP. Portions of Spn4p-GFP rings were photobleached, and the rates of recovery of Spn4p-GFP fluorescence were assayed. In *mid2*$^+$ cells, Spn4p-GFP in well established septin rings recovered relatively slowly with $t_{1/2}$ = 350 ± 136 s (range 168–532 s; *n* = 7) (Fig. 5; see Materials and methods), showing that Spn4p is relatively stable. In contrast, in *mid2Δ* cells Spn4p-GFP fluorescence recovered over 30-fold more rapidly, with $t_{1/2}$ = 10 ± 4 s (range 5–17 s; *n* = 6) (Fig. 5) showing that Spn4p is rapidly exchanging in the ring. Similar FRAP rates were observed in *mid2Δ* cells at all different stages of cleavage (*n* = 18), showing that this large difference between wild-type and *mid2Δ* cells was not due to cell cycle stage differences between the datasets. Since the rate of septin exchange is much faster than the rate of invagination, these dynamics show that Spn4p proteins in *mid2Δ* cells are not flowing in from the rings at the cell surface into the furrow but may be rapidly binding and exchanging with the invaginating membrane. Thus, Mid2p is required to stabilize septins.

Mid2p colocalizes with septins and is dependent on septins for localization

Next, we tested whether Mid2p colocalizes with septins. We examined the localization of functional Mid2p-GFP or

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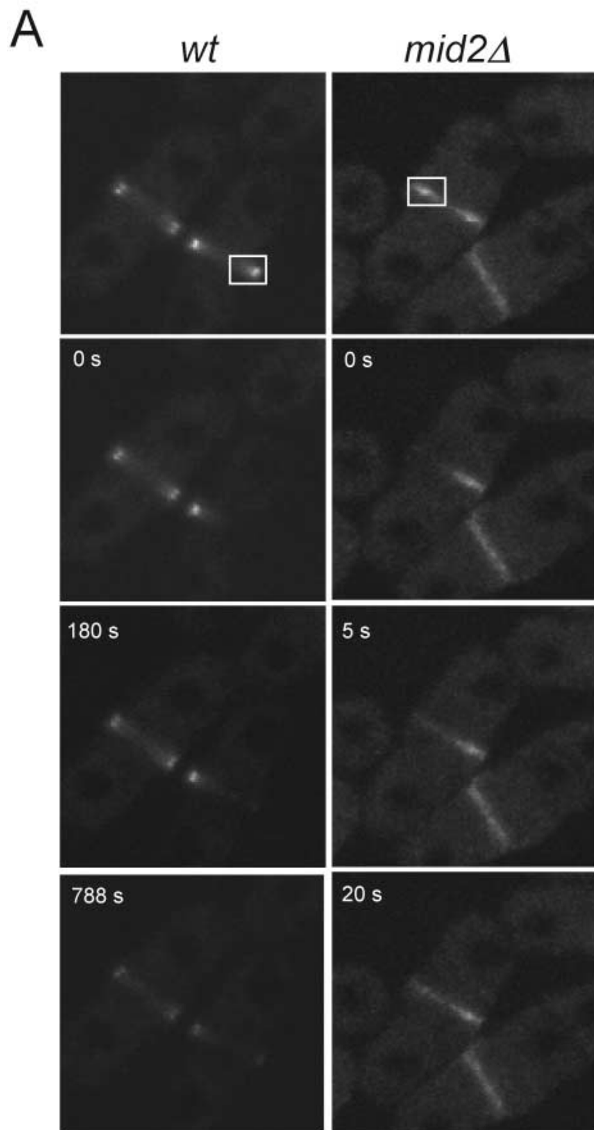

Figure 5. **Measurement of Spn4p-GFP dynamics using FRAP.** (A) Wild-type and *mid2Δ* cells expressing Spn4p-GFP were photobleached in the zones marked by white rectangles. Recovery of fluorescence intensity was assayed over time (as described in Materials and methods). Representative images are shown. Top panels show cells just before photobleaching. 0 s represents time immediately after photobleaching. (B) Graph of mean $t_{1/2}$ ± SD of Spn4p-GFP fluorescence recovery in seconds.

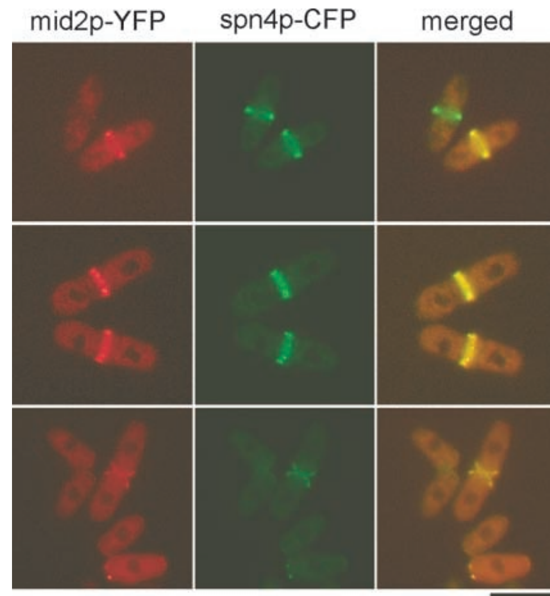

Figure 6. **Mid2p colocalizes with Spn4p.** Cells (FC882) expressing Mid2p-YFP (green) and Spn4p-CFP (red) were imaged for YFP and CFP fluorescence. (Top) Cells in late mitosis just after initial assembly of the single septin ring. Some of these cells exhibit septin but not Mid2p staining (left cell). (Middle) Cells during septation with double septin rings. (Bottom) Cells during cell–cell separation and during interphase. Bar, 10 µm.

Mid2p-YFP fusions expressed from the chromosomal endogenous *mid2*$^+$ promoter. Using dual labeling with Mid2p-YFP and Spn4p-CFP constructs, we found that Mid2p and Spn4p colocalized precisely in single and double rings during mitosis (Fig. 6). The two proteins colocalized even during interphase when they were sometimes localized in dots at the cell tip or in a single large cytoplasmic dot. 20% of mitotic or septating cells (n = 176) had Spn4p-CFP present in a single ring but had no detectable Mid2p-YFP (Fig. 6, top). No cells were found with Mid2p-YFP present but Spn4p-CFP absent. Since the cells exhibiting rings of Spn4p without Mid2p were in the initial stages of septin ring localization, these results show that Spn4p may precede Mid2p localization at the septin ring.

We also tested the dependence of Mid2p localization on septins. In *spn4Δ* cells, Mid2p-GFP was not localized to any specific structure (Fig. 7 A). Western blot showed that Mid2p-GFP was still expressed in *spn4Δ* cells (unpublished data). As described above, septins initially localize normally in *mid2Δ* cells. Thus, both the order of localization and localization dependence results suggest that septins first form a single medial ring and that Mid2p associates with the septin ring slightly later in the cell cycle.

The Mid2p PH domain is required for Mid2p function and localization

We tested the function of the conserved COOH-terminal PH domain of Mid2p. *mid2 PHΔ* and *mid2 PHΔ-YFP* mutants were generated by introducing a kanMX or YFP-kanMX cassette into the *mid2*$^+$ coding region to produce a COOH-terminal truncation. *mid2 PHΔ* cells had the same cell–cell separation phenotype as *mid2Δ* mutants (Fig. 7 B).

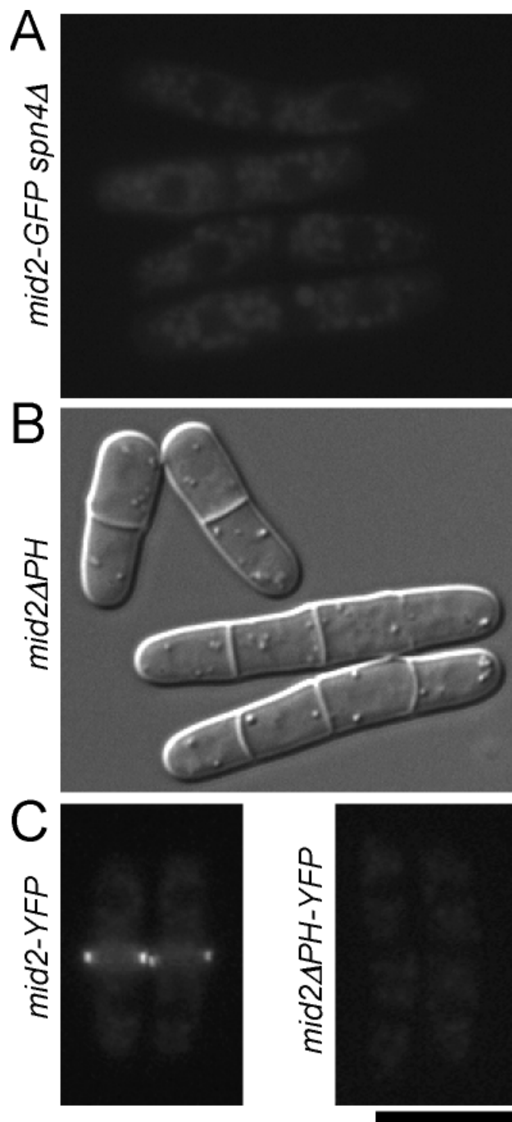

Figure 7. **Mid2p localization is dependent on septins and the Mid2p PH domain.** (A) *spn4Δ* cells (FC492) expressing Mid2p-GFP were imaged for GFP fluorescence. Only background fluorescence is observed. (B) Cells expressing only Mid2p-PHΔ were imaged by DIC microscopy. (C) *mid2-YFP* and *mid2-PHΔ–YFP* cells were imaged for YFP fluorescence. Only background fluorescence is observed in the *mid2-PHΔ–YFP* cells. Bar, 10 μm.

mid2 PHΔ-YFP cells exhibited only diffuse YFP fluorescence, showing that this truncated protein was not localized properly (Fig. 7 C). Western blotting confirmed that the Mid2p-PHΔ−GFP protein was still expressed (unpublished data). Similar results were found with a *mid2 PHΔ-HA* strain where the mutant was tagged with a HA epitope tag (unpublished data). Thus, the PH domain is required for Mid2p function and localization.

mid2^{+} does not share overlapping functions with *mid1*$^{+}$

Since Mid2p and Mid1p share significant amino acid similarity, we tested whether they may share overlapping functions. Wild-type, single *mid1Δ*, and *mid2Δ* mutants, and *mid1Δmid2Δ* double mutants were assayed for growth and cell integrity at multiple temperatures on agar plates containing phloxin, a red dye that stains dead cells. We also assayed for septum placement defects in cells grown in liquid cultures. In these assays, the phenotype of the *mid1Δmid2Δ* double mutant was not more severe than that of either single mutant (Fig. 8, A and B). Mid1p-GFP was properly localized as a medial broad band of dots at the cell surface and in a tight ring in *mid2Δ* mutant cells (Fig. 8 C) (Paoletti and Chang, 2000). Mid2p-GFP was properly localized in single or double rings in the *mid1Δ* mutant (Fig. 8 D). Thus, together our data suggest that Mid1p and Mid2p do not have overlapping functions and act in different aspects of cytokinesis.

Discussion

Mid2p stabilizes the septin ring

Here, we identified Mid2p, a protein necessary for efficient cell–cell separation during cytokinesis in fission yeast. Common phenotypes suggest that Mid2p and septins share a common function. Mid2p localizes to the cell division site after septins have been deposited and requires septins for localization. Although Mid2p is not required for the initial formation of the septin ring, it functions to maintain the integrity and stability of the septin rings during contractile ring closure. The PH domain of Mid2p, which may function as a membrane anchor or as a septin interaction domain, is required for the function and localization of the protein. These observations suggest that Mid2p functions primarily to organize septin rings at the plasma membrane and is required for proper septin function in cell–cell separation.

Septins are filament-forming proteins. In vitro, septins assemble into 10-nm filaments (Field et al., 1996; Frazier et al., 1998; Mendoza et al., 2002). In budding yeast, septins appear to be components of the bud neck filaments, although in other organisms analogous septin filaments in vivo have not yet been reported. Little is known about how septin filaments may be regulated by other protein factors, such as factors that promote polymerization, depolymerization, or filament bundling, or affect GTP hydrolysis or exchange. Here we show that in contrast to the contractile ring proteins actin, cdc4p, cdc8p, and myo2p which exchange rapidly (mean $t_{1/2} < 1$ min) (Pelham and Chang, 2002; Wong et al., 2002), septins form relatively stable structures in the ring (mean $t_{1/2} = 7$ min). We speculate that Mid2p promotes the assembly and/or the stabilization of septin filaments. The abnormally rapid dynamics of septins in *mid2Δ* cells may arise from unassembled septin proteins that exchange with binding sites at the membrane of the cleavage furrow. Expression of a nondegradable form of the Mid2p protein has been shown to stabilize septin rings so that they persist into the next cell cycle (K. Gould, personal communication), suggesting that Mid2p can prevent the disassembly of the septin ring at cell division. To our knowledge, our study presents one of the first in vivo analyses of septin dynamics using FRAP identifying a protein required specifically for septin stability.

Although other septin-interacting proteins have not been well characterized yet in *S. pombe*, a number of proteins have been identified that regulate septins in budding yeast.

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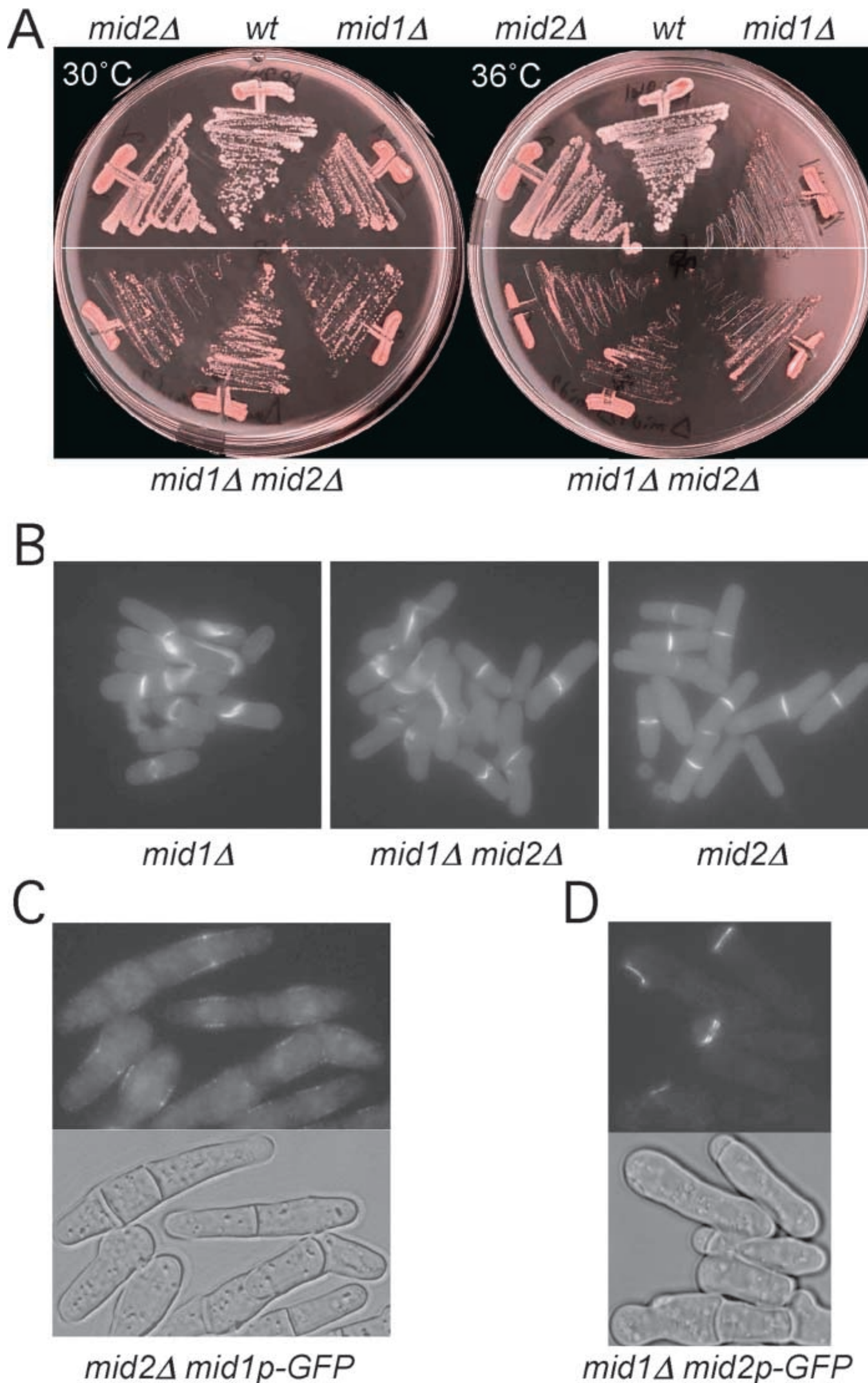

Figure 8. ***mid1* and *mid2* do not show genetic interactions.** (A) Wild-type, *mid1Δ*, *mid2Δ*, and *mid1Δmid2Δ* strains were assayed for growth rates and cellular integrity at 30 and 36°C by growth on agar plates containing phloxin, a dye that stains dead cells. Three independent *mid1Δmid2Δ* strains are represented in the bottom three streaks of each plate. (B) Cells grown in liquid cultures were stained with Calcofluor to visualize septa. (C) Localization of Mid1p-GFP in *mid2Δ* cells and localization of Mid2p-GFP in *mid1Δ* cells.

The GTPase Cdc42p is likely to be a central regulator of septin ring assembly in budding yeast (Gladfelter et al., 2002). Mutants of budding yeast Gin4p, a protein kinase that may directly interact with septins, have septin "bars" rather than rings (Longtine et al., 1998, 2000). *elm1* and specific *cdc42* mutant alleles display misplacement of septin rings (Bouquin et al., 2000; Gladfelter et al., 2002). Septins have been found to be conjugated with the ubiquitin-related protein SUMO and interact with components of the sumoylation machinery, such as the E3-like factor Siz1p (Johnson and Blobel, 1999; Takahashi et al., 1999; Johnson and Gupta, 2001).

Mid2p and septin function in *S. pombe*

Septins have been implicated in cytokinesis in several organisms. In budding yeast, septins are essential for cytokinesis and for other important functions such as regulation of the cell cycle and cell shape (Gladfelter et al., 2001). Surprisingly, septin mutants in fission yeast have a much milder phenotype in which many aspects of cytokinesis such as actomyosin ring assembly and contraction and septation are apparently normal, and only the final step in cell–cell separation is delayed for about a generation time (2–3 h). This step in cytokinesis may involve the deposition or activation of factors at the septum that degrade the primary septum, allowing for cell sepa-

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ration. What may be the functions of septins in cytokinesis in *S. pombe*? Other mutants with a similar cell–cell separation phenotype include mutants in the exocyst complex (*sec6*, *sec8*, *sec10*, and *exo70*), calcineurin (*ppb1*), a MAPK (*pmk1*), a MAPK phosphatase (*pmp1*), and a forkhead transcription factor (*sep1*) (Sipiczki et al., 1993; Yoshida et al., 1994; Toda et al., 1996; Ribar et al., 1997; Sugiura et al., 1998; Wang et al., 2002). Possible effects of these gene products on Mid2p and septins remain to be determined. In other organisms, septins interact with the exocytosis machinery, such as exocyst components or syntaxins (Hsu et al., 1998; Beites et al., 1999; Kartmann and Roth, 2001). Thus, septins and Mid2p may be required for proper exocytosis of a septum digestive enzyme to the septum.

Septins are still able to localize normally to medial plasma membrane even in the absence of Mid2p. This distribution suggests that there are septin-binding sites in this region. Filipin staining shows that an oxysterol-rich membrane domain is established in a medial band starting in anaphase, and then in the region of the cleavage furrow during cytokinesis (unpublished data). Formation of this membrane domain is independent of septins and Mid2p (unpublished data). Since septins may bind directly to phospholipids (Zhang et al., 1999), they may initially recognize and bind to this membrane domain before cleavage. One function of Mid2p may be to stabilize septins so that they stay in rings at the cell surface and do not associate with the rest of the membrane domain in the interior of the cleavage furrow.

New family of cytokinesis proteins

Mid2p has significant similarity to several other proteins involved in cytokinesis and/or septin association: *S. pombe* Mid1p, *S. cerevisiae* Bud4, and *C. albicans* Int1 (Gale et al., 1996; Sanders and Herskowitz, 1996; Sohrmann et al., 1996). These proteins begin to define a new family of cytokinesis proteins. All share a very COOH-terminal PH domain and have additional areas of similarity at the COOH terminus. Mid1p is a protein involved in the positioning of the actomyosin contractile ring, since *mid1* mutants form rings in random locations at the cell surface. No association between Mid1p and septins have been noted. Genetic tests did not reveal any overlapping functions between Mid1p and Mid2p. Rather, Mid1p and Mid2p may function independently at two different parts of the cell cycle: Mid1p acts in early mitosis to organize and position the actomyosin ring, whereas Mid2p acts in late mitosis to organize the septin rings.

Bud4p and Int1p influence the placement of the cell division site and also appear to associate with the septins. Budding yeast Bud4p is located at the septin rings at the bud neck and functions in positioning the future bud site at a site adjacent to the septin ring from the previous cell cycle (Sanders and Herskowitz, 1996). Although septin rings appear normal in *bud4* mutants, Bud4p does not appear to be involved in organizing the septin structure but may use septins as a means for localization. *C. albicans* Int1p is required for hyphal formation, adherence, and pathogenesis (Gale et al., 1998). Overexpression of Int1p in budding yeast causes reorganization of the septins into spiral-like structures, and Int1p coimmunoprecipitates with budding yeast septins (Gale et al., 2001). In *Candida*, Int1p colocalizes with a septin ring distal to the germ tube neck and is required for bud site selection.

The functional metazoan homologues of these proteins may be anillins, contractile ring proteins identified in flies, *Xenopus,* and human (Field and Alberts, 1995; Oegema et al., 2000). Anillins also have a COOH-terminal PH domain, but the amino acid similarity outside of the PH domain to the fungal proteins is low. Recent in vivo and in vitro results show that anillin may function directly to link septins to actin bundles (Oegema et al., 2000; Kinoshita et al., 2002). The COOH-terminal region of anillin that encompasses the PH domain is required for this septin recruitment activity and for its localization to the cleavage furrow. Thus, the COOH-terminal portion of all these proteins, including Mid2p, may share a common function in septin interaction. Since anillin associates with both actin and septins, its function may encompass both the roles of Mid1p and Mid2p. Additional proteins related to anillin are also encoded in the genomes of *Drosophila*, *Caenorhabditis elegans*, human, and mice, suggesting the possibility that these proteins have diverse or additional molecular functions (Oegema et al., 2000). Therefore, these fungal and metazoan proteins may serve conserved functions in cytokinesis as important organizers of cleavage furrow components.

Materials and methods

Yeast genetic, biochemical, and cell biological methods

S. pombe strains used in this study are listed in Table I. Standard methods for *S. pombe* media, genetic manipulations, immunofluorescence, and staining are described at http://www.bio.uva.nl/pombe/handbook/. Construction of *mid2Δ, mid2p-GFP, mid2p-YFP, mid2p-HA, spn4-CFP, and cdc12-CFP* strains was performed using a PCR-based approach using kanMX-based templates and 100-mer oligos with 80-bp homologies to the targeted gene (Bahler et al., 1998b; Glynn et al., 2001) (Yeast Resource Center). *spn4Δ::kanMX* and *spn4-GFP* strains were gifts from J. Pringle (University of North Carolina, Chapell Hill, NC) and were constructed using a similar approach. For generation of *mid2Δ*, initial deletions were made in diploid strains, and then heterozygous diploids were sporulated to produce viable *mid2Δ* haploid spores. Subsequent deletions and insertions were performed in haploid strains. Deletion and insertion strains were confirmed by PCR to check proper insertion at the locus.

Microscopy

Microscopy was performed using wide field or spinning disk confocal light microscopy (Pelham and Chang, 2002) using Open Lab 2 (Improvision) software for image acquisition and analysis. For three-dimensional confocal images of contractile or septin rings, cells were grown in log phase cultures (diluted from overnight cultures 3 h before imaging), pelleted, placed onto agarose pads (Tran et al., 2001), and imaged in 17 image planes 0.5 μm apart every 4 min. Images were reconstructed in three dimensions using a three-dimensional module with linear interpolation for addition of 1 plane between each slice. FRAP studies were performed on a Zeiss LSM510 two-photon scanning confocal and analyzed using Zeiss LSM software (Carl Zeiss MicroImaging, Inc.) as described (Pelham and Chang, 2002). Fluorescence intensities were normalized to a control unbleached ring in the same field to account for photobleaching from image acquisition after the initial photobleach. Alexa phalloidin staining was performed as described (Pelham and Chang, 2001). Spn4p immunofluorescence was performed on methanol-fixed cells with anti-Spn4p antibody (a gift from J. Pringle, University of North Carolina, Chapel Hill, NC) as described in http://www.bio.uva.nl/pombe/handbook/.

Western analysis

Western blotting was performed with yeast extracts prepared using a mortar and pestle method (Glynn et al., 2001). Anti-GFP antibodies (from J. Kahana and P. Silver, Harvard Medical School, Boston, MA, or K. Sawin,

Table I. ***S. pombe* strains used in this study**

Strain	Genotype	Source
FC881	*h⁻ mid2Δ::kanMX6 ade6 leu1-32 ura4-D18*	This work
FC937	*h⁻ ade6 leu1-32 ura4-D18*	This work
FC866	*h⁻ spn4-GFP-kanMX6*	J. Pringle
FC867	*h⁻ spn4Δ::kanMX6 leu1-32 ura4-D18*	J. Pringle
FC940	*h⁻ mid2Δ::kanMX6 spn4-GFP-kanMX6*	This work
FC941	*h⁺ mid2-GFP-kanMX6 ade6 leu1-32 ura4-D18*	This work
FC880	*h⁺ mid2-YFP-kanMX6 ade6 leu1-32 ura4-D18*	This work
FC882	*h⁺ mid2-YFP-kanMX6 spn4-CFP-kanMX leu-32*	This work
FC942	*h⁻ mid2-GFP-kanMX6 spn4Δ::kanMX6 ade6 ura4-D18*	This work
FC943	*h⁻ mid2 PHΔ-YFP-kanMX6 ade6 leu1-32 ura4-D18*	This work
FC944	*h⁻ mid2 PHΔ-kanMX6 ade6 leu1-32 ura4-D18*	This work
FC936	*h⁺ mid2 PHΔ-HA-kanMX6 ade6 leu1-32 ura4-D18*	This work
FC945	*h⁻ mid2 PHΔ-HA-kanMX6 spn4Δ::kanMX6 ade6 leu1-32 ura4-D18*	This work

University of Edinburgh, Edinburgh, UK) were used at a 1:1,000 dilution. Anti-HA antibodies (Covance) were used at a 1:1,000 dilution.

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