An anillin homologue, Mid2p, acts during fission yeast cytokinesis to organize the septin ring and promote cell separation

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An anillin is a conserved protein required for cell division (Field, C.M., and B.M. Alberts. 1995. J. Cell Biol. 131:165–178; Oegema, K., M.S. Savoian, T.J. Mitchison, and C.M. Field. 2000. J. Cell Biol. 150:539–552). One fission yeast homologue of anillin, Mid1p, is necessary for the proper placement of the division site within the cell (Chang, F., A. Woollard, and P. Nurse. 1996. J. Cell Sci. 109(Pt 1):131–142; Sohrmann, M., C. Fankhauser, C. Brodbeck, and V. Simanis. 1996. Genes Dev. 10:2707–2719). Here, we identify and characterize a second fission yeast anillin homologue, Mid2p, which is not orthologous with Mid1p. Mid2p localizes as a single ring in the middle of the cell after anaphase in a septin- and actin-dependent manner and splits into two rings during septation. Mid2p colocalizes with septins, and mid2Δ cells display disorganized, diffuse septin rings and a cell separation defect similar to septin deletion strains. mid2 gene expression and protein levels fluctuate during the cell cycle in a sept1- and Skp1/Cdc53/F-box (SCF)–dependent manner, respectively, implying that Mid2p activity must be carefully regulated. Overproduction of Mid2p depolarizes cell growth and affects the organization of both the septin and actin cytoskeletons. In the presence of a nondegradable Mid2p fragment, the septin ring is stabilized and cell cycle progression is delayed. These results suggest that Mid2p influences septin ring organization at the site of cell division and its turnover might normally be required to permit septin ring disassembly.

Introduction

Cytokinesis is the final stage of mitosis, during which a cell is irreversibly split into two daughters. In many eukaryotic organisms, an essential element of this process is an actomyosin-based contractile ring whose constriction is required to generate the force necessary to complete cell division. Schizosaccharomyces pombe is an excellent organism for studying cytokinesis, as it divides by medial fission (Field, C.M., and B.M. Alberts. 1995. J. Cell Biol. 131:165–178; Oegema, K., M.S. Savoian, T.J. Mitchison, and C.M. Field. 2000. J. Cell Biol. 150:539–552). One fission yeast homologue of anillin, Mid1p, is necessary for the proper placement of the division site within the cell (Chang, F., A. Woollard, and P. Nurse. 1996. J. Cell Sci. 109(Pt 1):131–142; Sohrmann, M., C. Fankhauser, C. Brodbeck, and V. Simanis. 1996. Genes Dev. 10:2707–2719). Here, we identify and characterize a second fission yeast anillin homologue, Mid2p, which is not orthologous with Mid1p. Mid2p localizes as a single ring in the middle of the cell after anaphase in a septin- and actin-dependent manner and splits into two rings during septation. Mid2p colocalizes with septins, and mid2Δ cells display disorganized, diffuse septin rings and a cell separation defect similar to septin deletion strains. mid2 gene expression and protein levels fluctuate during the cell cycle in a sept1- and Skp1/Cdc53/F-box (SCF)–dependent manner, respectively, implying that Mid2p activity must be carefully regulated. Overproduction of Mid2p depolarizes cell growth and affects the organization of both the septin and actin cytoskeletons. In the presence of a nondegradable Mid2p fragment, the septin ring is stabilized and cell cycle progression is delayed. These results suggest that Mid2p influences septin ring organization at the site of cell division and its turnover might normally be required to permit septin ring disassembly.

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identified in budding yeast (Hartwell, 1971) and now described in many organisms (for review see Faty et al., 2002; Macara et al., 2002). In *Saccharomyces cerevisiae*, septins are thought to perform multiple functions at the mother–daughter bud neck, such as providing a boundary that restricts certain determinants to particular cortical domains (for review see Faty et al., 2002) and acting as a scaffold necessary for the proper localization of many factors involved in polarity and cell division (for review see Gladfelter et al., 2001). Whether septins perform all of these roles in other organisms has yet to be established. However, most septins studied to date localize to the site of cell division, and disruption of septin function leads to cytokinesis defects in many cell types (Hartwell, 1971; Neufeld and Rubin, 1994; Longtine et al., 1996; Kinoshita et al., 1997).

One factor important for septin organization is the conserved protein anillin, which is concentrated in the cleavage furrow of dividing cells where it links the actin and septin cytoskeletons (Field and Alberts, 1995; Oegema et al., 2000; Kinoshita et al., 2002). Further, the disruption of anillin function leads to defects in cytokinesis (Oegema et al., 2000). In *S. pombe*, there is no evidence that the one described anillin homologue, Mid1p, interacts with the septin cytoskeleton. Rather, the loss of Mid1p function results in misplaced actomyosin rings and septa (Chang et al., 1996; Sohrmann et al., 1996; Bähler et al., 1998a; Paoletti and Chang, 2000), whereas a septin deletion results in a cell separation defect (J. Pringle, personal communication; Longtine et al., 1996). Thus, these proteins appear to be involved in different stages of cell division.

Examination of the recently completed *S. pombe* genome sequence indicated the presence of a second ORF with significant homology to Hs anillin and mid1 that we have termed mid2 (Q9P7Y8 or SPAPYUG7.03C). In this study, we have examined the functional relationship of Mid2p with both Mid1p and the septins. Mid2p is regulated in abundance during the cell cycle, and we have examined the reasons for its periodicity. Our results suggest that Mid2p plays a role during late mitosis in organizing the septin ring and that Mid2p degradation may facilitate septin ring disassembly.

**Results**

*S. pombe* mid2 encodes a second anillin homologue

Examination of the *S. pombe* protein database identified an uncharacterized protein with homology to Mid1p that has been termed Mid2p (Q9P7Y8 or SPAPYUG7.03C). In this study, we have examined the functional relationship of Mid2p with both Mid1p and the septins. Mid2p is regulated in abundance during the cell cycle, and we have examined the reasons for its periodicity. Our results suggest that Mid2p plays a role during late mitosis in organizing the septin ring and that Mid2p degradation may facilitate septin ring disassembly.
Mid2p localization and abundance are cell cycle regulated

To better understand the role of Mid2p in cell separation, its subcellular localization was examined. A carboxy-terminal Mid2p–GFP fusion protein was constructed by homologous recombination at the endogenous locus of mid2+ in order to maintain its physiological expression level. Mid2p–GFP localized to the medial region in 17% of the cells in an asynchronous population (Fig. 2 A). Using time-lapse microscopy, Mid2p–GFP was observed to initially form a single ring, split into two rings as the septum formed, and then disappear as cells separated (see Video 1, available at http://www.jcb.org/cgi/content/full/jcb.200211126/DC1). Mid2p–GFP was stable to a variety of fixation procedures, thereby permitting it to be visualized in fixed cells also stained with DAPI and antibodies to tubulin. In this case, Mid2p was only detected at the ring in late anaphase cells that lacked spindles (Fig. 2 B; unpublished data).

Because Mid2p–GFP localization was transient, we asked whether Mid2p levels were cell cycle regulated. A Mid2p–HA strain was constructed and used to examine protein levels in a culture synchronized by centrifugal elutriation. Cell samples were collected at the indicated times after synchronization and processed for protein (C) or RNA (E). (C) Cell cycle progression was monitored by determining septation index and Cdc13p levels. Mid2p–HA, Cdc13p, and Cdc2p (which served as a loading control in all of our immunoblots) were detected by antibodies, respectively. (D) Mid2p–HA (KGY2843) was immunoprecipitated with 12CA5 antibodies and incubated with antibodies to tubulin. This in case, Mid2p was only detected at the ring in late anaphase cells that lacked spindles (Fig. 2 B; unpublished data).

Because Mid2p–GFP localization was transient, we asked whether Mid2p levels were cell cycle regulated. A Mid2p–HA strain was constructed and used to examine protein levels in a culture synchronized by centrifugal elutriation. Mid2p–HA levels fluctuated considerably as cells traversed the cell cycle, peaking concomitantly with septation (Fig. 2 C). Also, Mid2p–HA appeared to migrate as several distinct species (Fig. 2 C). These multiple isoforms are due to phosphorylation because treatment of immunoprecipitated Mid2p–HA with λ-phosphatase collapsed them into a single, faster-migrating species (Fig. 2 D).

To determine if Mid2p periodicity was due, at least in part, to fluctuations in its mRNA, mid2 transcript levels were monitored from a synchronous culture of wild-type cells harvested by centrifugal elutriation. Asynchronous population (Fig. 2 A) (Lemmon and Ferguson, 2001). Sporulation and tetrad dissection of diploids that were confirmed to have the entire mid2 ORF successfully replaced by ura4+ produced four viable colonies, indicating that mid2 is not essential for viability. In contrast to the misplaced septum phenotype observed in mid1Δ cells, the loss of mid2 produced “chained” cells in which repeated rounds of nuclear division and septum synthesis occurred with delayed cell separation (Fig. 1 B). To determine if Mid1p and Mid2p share an overlapping essential function, a mid1Δ mid2Δ strain was constructed. This strain was viable at 25°C, exhibited both septum placement and cell separation defects (Fig. 1 B), and was temperature sensitive (unpublished data), as is the mid1Δ strain alone. Combined with the fact that both Mid1p–GFP and Mid2p–GFP were able to localize properly in the absence of the other (unpublished data), we conclude that mid1 and mid2 are not functionally redundant.

Mid2p is destroyed by Skp1/Cdc53/F-box (SCF)–dependent proteolysis

Because of the dynamic changes in Mid2p–HA levels during the cell cycle (Fig. 2 C), we tested if Mid2p was targeted for destruction by ubiquitin-mediated proteolysis. First, we examined whether Mid2p was ubiquitinated in vivo. In an mts3–1 mutant strain, multi-ubiquitinated conjugates accumulate at the restrictive temperature due to the abrogation of proteosome function (Gordon et al., 1996). Therefore, polyubiquitinated Mid2p–Myc would be expected to accrue in an mts3–1 strain, but only if the relevant E3 ubiquitin ligase was active. S. pombe securn, Cut2p, was used as a positive control for a ubiquitin-conjugated protein (Berry et al., 1999). Both Cut2p–Myc and Mid2p–Myc were readily de-
Rather, Mid2p–Myc ubiquitination was strictly dependent upon functional Skp1p (Fig. 3 A, lanes 7 and 9), whereas Cut2p–Myc proteolysis was SCF independent (Fig. 3 A, lanes 1 and 3). An anti-ubiquitin immunoblot confirmed that ubiquitin conjugates were purified in all strains except the one lacking the His–ubiquitin vector (Fig. 3 B).

Consistent with the in vivo ubiquitination assay data, Mid2p–HA levels accumulated in an mts3-1 strain (Fig. 3 C) but not in an APC mutant at the restrictive temperature (unpublished data). Mid2p–HA abundance was also elevated in cells lacking two F-box–encoding genes, pop1 and pop2, components of SCF ubiquitin ligases (Fig. 3 C) (Kominami et al., 1998). Taken together, these results are consistent with the hypothesis that Mid2p is destroyed by SCF-dependent ubiquitination in vivo.

**mid2 expression is dependent on the forkhead-like transcription factor sep1**

The fact that mid2 mRNA levels oscillate during vegetative growth (Fig. 2 E), coupled with the observation that the mid2Δ cell separation defect resembles the loss of a putative transcription factor, sep1 (Fig. 4 A; Ribar et al., 1999), prompted us to examine whether mid2 transcript abundance depended on Sep1p function. A cdc25-22 block and release synchronization protocol (Moreno et al., 1990) was used to address this question because the sep1Δ strain cannot be syn-

![Figure 3](image-url)

**Figure 3. Mid2p is regulated by SCF-dependent proteolysis.** (A and B) In vivo ubiquitination assays. The cut2–myc mts3-1 (KGY1923) (lane 1), cut2–myc mts3-1 lid1-6 (KGY1948) (lane 2), cut2–myc mts3-1 skp1-A4 (KGY4050) (lane 3), mts3-1 (KGY574) (lane 5), mid2–myc (KGY2432) (lane 6), mid2–myc mts3-1 (KGY3687) (lane 7), mid2–myc mts3-1 lid1-6 (KGY1977) (lane 8), and mid2–myc mts3-1 skp1-A4 (KGY1978) (lane 9) strains containing pREP1-His–ubiquitin (Ub) and the mid2–myc mts3-1 strain (KGY3687) without vector (lane 4) were grown at 25°C for 22 h in the absence of thiamine to induce His–Ub expression and then shifted to 36°C for 4 h. Ub-conjugated proteins were isolated and immunoblotted with anti-HA (12CA5) to detect Mid2p–HA and anti-PSTAIR to detect Cdc2p.

![Figure 4](image-url)

**Figure 4. mid2 expression depends on Sep1p.** (A) Differential interference contrast images of mid2Δ (KGY3135) and sep1Δ (KGY3419) cells grown in YE medium at 32°C. Bar, 5 μm. (B–E) cdc25-22 mid2–HA (KGY3306) (B and D) and cdc25-22 mid2–HA sep1Δ (KGY3457) (C and E) cells were arrested in G2 by shift to 36°C for 4 h. Cultures were then released to 25°C and cell pellets collected at the indicated times to analyze Mid2p–HA and mid2Δ transcript levels. (B and C) Mid2p–HA and Cdc2p levels were determined by immunoblotting with 12CA5 and anti-PSTAIR, respectively. Mitotic progression was determined by assaying Cdc13p fluctuations by immunoblotting. (D and E) Northern blot analysis of mid2Δ transcript levels from the same time points analyzed in B and C. The his3 transcript served as a loading control.
chronized by centrifugal elutriation due to its chained cell phenotype (Ribar et al., 1999). In these cells, progression through mitosis was monitored by examining fluctuations in Cdc13p–cyclin B levels that occur during this period (Alfa et al., 1989). Although readily detectable in cdc25-22 cells proceeding through mitosis (Fig. 4 B), Mid2p–HA levels were clearly diminished in sep1Δ cells during the same time course (Fig. 4 C). The drop in Mid2p levels correlated with a significant decrease in mid2 mRNA abundance in the absence of sep1, although delayed and reduced amounts of the mid2 transcript were detected (Fig. 4, D and E). These results indicate that Sep1p plays an important role in setting Mid2p levels.

To address whether mid2Δ might be the critical target of Sep1p, we tested whether expression of mid2Δ from a heterologous promoter was able to suppress the cell separation defect of a sep1Δ strain. At best, we observed a partial rescue (unpublished data), indicating that other targets of Sep1p must exist that, together with Mid2p, are required for efficient cell separation in S. pombe.

Medial ring localization of Mid2p–GFP requires F-actin
To determine if Mid2p required F-actin for its association with the medial ring, Mid2p–GFP localization was monitored in cells released from a cdc25-22 block into either DMSO or latrunculin A (LatA), which promotes F-actin depolymerization (Ayscough et al., 1997). In control cells, the first Mid2p–GFP rings appeared at 60 min and persisted (Fig. 5 A; unpublished data). In contrast, the LatA-treated cells failed to form Mid2p–GFP rings even after 150 min (Fig. 5 A). In an asynchronous culture of cells, however, Mid2p–GFP persisted at the medial ring 15 min after LatA addition (Fig. 5 B), suggesting that once recruited, Mid2p’s association with the medial ring is actin independent.

Mid2p is required for proper septin ring organization
Because the loss of mid2 produced a strikingly similar phenotype to that of the spo4Δ mutant (Fig. 5 C) (J. Pringle, personal communication; Longtine et al., 1996), and the phenotype of a mid2Δ spo4Δ double mutant strain was similar to either single mutant (Fig. 5 C), it seemed likely that Mid2p and septins were involved in the same step of cell division. We therefore examined if Mid2p depended upon septins for its medial ring association. In spo4Δ cells, Mid2p–GFP was expressed (unpublished data) but failed to localize to the medial ring and was instead distributed throughout the cytoplasm (Fig. 5 D). We next determined whether or not septins and Mid2p colocalized. Images taken from a strain expressing endogenously tagged Mid2p–GFP and Spn1p–CFP show that their localization patterns are indistinguishable (Fig. 6 A). We also tested whether septin localization was affected by the lack of Mid2p function. To perform this experiment, the spo3Δ locus was modified to encode an Spn3p–GFP fusion protein. This strain was morphologically wild type, implying that the epitope does not disrupt septin function. By time-lapse microscopy, Spn3p–GFP was observed to form a single ring, then form two rings, and finally disperse upon the completion of cell separation (Fig. 6 B; see Video 2, available at http://www.jcb.org/cgi/content/full/jcb.200211126/DC1). In a mid2Δ strain, Spn3p–GFP was recruited to the site of cell division in a loosely organized ring and then appeared to spread bilaterally across the septum as a disc over time (Fig. 6 C; see Video 3, available at http://www.jcb.org/cgi/content/full/jcb.200211126/DC1).

Because septa persist in a mid2Δ strain, we wondered whether the abnormal Spn3p–GFP localization was a direct consequence of the loss of mid2 or a byproduct of the cell separation defect in these cells. Therefore, we examined Spn3p–GFP localization in a different mutant that also displays multiple uncleaved septa. In the calcineurin deletion,
ppb1Δ, which displays this phenotype (Yoshida et al., 1994). Spn3–GFP assembled into tight rings at the cell cortex (Fig. 6 D), as did Mid2p–GFP (Fig. 6 E). This observation indicates that septin rings are not necessarily disorganized in cell separation mutants but are specifically affected by the absence of Mid2p.

**Delineation of Mid2p functional domains**

To determine which regions of the protein dictated Mid2p localization and were important for its function, a series of mid2 constructs were generated and tested for their ability to rescue the mid2Δ cell separation defect, their localization pattern, and any overexpression phenotype (Fig. 7 B). Immunoblot analysis of anti-HA immunoprecipitations from denatured lysates confirmed that these fragments were all produced (Fig. 7 C).

The PH domain of Mid2p encompasses amino acids 582–685 (Fig. 7 A). A construct lacking most of this motif (Mid2p 1–595) does not rescue the mid2Δ phenotype or localize to the medial ring. However, two constructs containing the PH domain (Mid2p 569–704 and Mid2p 596–704) failed to direct GFP to the medial ring or rescue the null phenotype (Fig. 7 B; unpublished data). Therefore, the PH domain appears to be necessary, but not sufficient, for Mid2p localization and function. Indeed, the smallest fragment directing medial ring localization was Mid2p (336–704) (Fig. 7 D), although it also seemed to concentrate in or around the nucleus. Mid2p (336–704) was also able to partially rescue the mid2Δ phenotype.

In contrast to the denatured lysates analyzed directly in Fig. 2 C and Fig. 4 B, we noticed that HA–Mid2p was degraded during an immunoprecipitation procedure to produce a variety of smaller fragments (Fig. 7 C). However, deletion of the Mid2p amino terminus, which contains three putative PEST (Pro/Glu/Ser/Thr) domains that are predicted to contribute to protein instability by facilitating ubiquitin-mediated proteolysis (Rogers et al., 1986), largely prevented this degradation (Fig. 7 C). Coupled with the fact that Mid2p is multi-ubiquitinated in vivo (Fig. 3 A), these...
observations suggest that the putative PEST domains contribute to Mid2p instability.

To test whether deletion of the PEST motifs would yield a stable version of Mid2p, either full-length Mid2p or Mid2p (336–704) was produced in mid2Δ cdc25-22 cells under control of the moderate nmt41 promoter as amino-terminal HA fusion proteins. After 22 h at 25°C in the absence of thiamine, these cells were shifted to 36°C to block cells at G2/M before maximal expression was reached. The cells were then released to 25°C in the presence of thiamine to prevent further transcription, and samples were taken periodically for immunoblot analysis. As with endogenous Mid2p, the full-length fusion protein was degraded as cells septated and began a new cell cycle (Fig. 7 E). In contrast, Mid2p (336–704) remained unmodified and significantly more stable (Fig. 7 F).

Overproduction of Mid2p leads to ectopic septin structures

Given that mid2Δ cells had disorganized septin rings, we tested if excess amounts of wild type or the stable form of Mid2p would affect the septin cytoskeleton. Cells overproducing full-length Mid2p from the strong nmt1 promoter grew very slowly, became rounded, and had depolarized actin patches (Fig. 8 A), but did form colonies (unpublished data). In these cells, Spn3p–GFP was detected in aberrant filamentous structures that lacked F-actin, and very few cells were observed undergoing cytokinesis (Fig. 8 A). Overproduction of stable Mid2p (amino acids 336–704; Fig. 7 F) produced slightly elongated, rather than depolarized, cells that also grew very slowly (Fig. 8 A; unpublished data). In this case, septin rings did not disassemble on schedule. Instead, septin rings persisted at the new ends of cells well into the next cell cycle, and prominent remnants of septin structures could even be detected into a third cell division because they were observed at both tips in addition to the middle of the cell (Fig. 8 A).

To quantify this effect, five patterns of Spn3p–GFP localization were examined (Fig. 8 B). Septins are normally observed diffusely at the tips of interphase cells, but for the purpose of this quantification, tip localization was only considered if it was of equal intensity to the ring fluorescence. In the vector control, 83% of cells lacked Spn3p–GFP staining at the ring or tips while 17% of the cells contained a septin ring(s) at the center of the cell (n = 2,372 cells; Fig. 8 B, 1 and 2). By comparison, in the presence of stable Mid2p (336–704), the number of cells lacking Spn3p–GFP staining dropped to 33% while cells with only medial ring staining dropped to 9% (n = 1,982 cells; Fig. 8 B, 1 and 2). When Mid2p (336–704) was overproduced, the most prevalent pattern of Spn3p–GFP fluorescence was at one or both ends (32%; Fig. 8 B, 3). A small percentage of cells, 7%, exhibited Spn3p–GFP localization at the center of the cell in addition to the tips, implying that septin structures had persisted during one to two extra cell divisions (Fig. 8 B, 4). Finally, 19% of the cells overproducing Mid2p (336–704) had depolarized septin patches distributed throughout the cell (Fig. 8 B, 5). These three phenotypes were rarely observed in the vector control cells (<0.5%; Fig. 8 B, 3–5). Taken together, these data suggest that the carboxy terminus of Mid2p stabilizes septin ring structures and that the timely destruction of Mid2p facilitates the disassembly of septin rings normally observed during each cell cycle.

Discussion

In this study, we have characterized a novel S. pombe protein, Mid2p, related to the cell division site placement factor, Mid1p. Our results suggest that Mid2p is involved in the establishment of the septin ring that permits efficient cell division.
sequence alignments indicate that Mid1p (921 amino acids) is the possible actin-interacting region noted above and an
2000). The regions of particular sequence similarity include
similarity not only with each other but with human anillin.
mediating their unique roles during cytokinesis.
2000). Thus, the amino terminus of Mid1p and the carboxy
terminus of Mid2p are likely to be the key determinants in
whether or not either Mid1p or Mid2p interacts directly
actin filaments. Mid1p and Mid2p both contain PEST
mechanisms conferring protein instability and similar carboxy-
terminal regions that include a PH domain (≈36%). Although
PH domains can mediate the protein–phospholipid interactions
necessary to localize proteins to the plasma membrane
(for review see Lemmon and Ferguson, 2001), this region of
Mid2p is not sufficient to directly GFP to the cell cortex nor is
it required for Mid1p localization to the medial ring (this
study; Paoletti and Chang, 2000). Moreover, the Mid2p PH
domain lacks the consensus sequence that predicts direct
and avid binding to phosphoinositides (Isakoff et al., 1998).
Thus, the PH domains of these proteins may be involved in
protein–protein interactions. In the case of Mid2p, this region
is critical for its localization and function, and it is
 tempting to speculate that it mediates interaction with septins,
a hypothesis currently being explored. In the case of
Mid1p, the carboxy-terminal region including the PH domain
is dispensable for its function (Paoletti and Chang, 2000).
Thus, the amino terminus of Mid1p and the carboxy
terminus of Mid2p are likely to be the key determinants in
mediating their unique roles during cytokinesis.

As mentioned above, Mid1p and Mid2p share sequence
similarity not only with each other but with human anillin.
Interestingly, there are multiple uncharacterized anillin ho-
mologues in Drosophila and C. elegans (Oegema et al.,
2000). The regions of particular sequence similarity include
the possible actin-interacting region noted above and an
≈200–amino acid stretch that includes the PH domain.

Sequence alignments indicate that Mid1p (921 amino acids) is
more similar to the larger anillin family members, such as
Hs anillin (1,126 residues), Drosophila anillin (1,201 resi-
dues), and the C. elegans gene product Y49E10.19 (1,205
residues). Conversely, Mid2p more closely resembles the
smaller gene products, C. elegans Y43F8C.14 and Drosophila
CG4530. Indeed, the characterized anillin family members in
Drosophila and human seem to be more related to Mid1p
than Mid2p in their localization pattern and loss of function
phenotypes, with the exception of a connection to septins
(Field and Alberts, 1995; Oegema et al., 2000). As these
anillin homologues in higher eukaryotes are characterized, it
will be interesting to learn whether they all function in cy-
tokinesis. Further, it will be interesting to ascertain if they
are functionally redundant or have distinct roles during cy-
tokinesis as Mid1p and Mid2p do in S. pombe.

Several lines of evidence suggest that Mid2p affects septin
ring organization and stability. In the absence of Mid2p,
Spn3p–GFP is organized into a loose, rather than a tight,
ring that disperses bilaterally across the septum as it forms.
Conversely, in cells overproducing a stabilized Mid2p frag-
ment, septin rings, or remnants thereof, persisted one to two
cell divisions after they were formed. This observation im-
plies that the turnover of full-length Mid2p might normally
permit the timely disassembly of the septin ring. Further-
more, that these cells grew very slowly and were elongated
upon the overproduction of nondegradable Mid2p indicates
that septin ring disassembly may be important for normal
cell cycle progression. In support of this possibility, cells
producing a mutant form of the S. cerevisiae septin, Cdc3p,
in which two Cdk sites have been altered to alanine, display
two septin rings in G1 (Tang and Reed, 2002). Signifi-
cantly, these cells are delayed in cell cycle progression until
the old septin ring is disassembled (Tang and Reed, 2002).

In an independent study by Berlin et al. (2003), further evi-
dence that Mid2p affects septin ring organization was obtained
by FRAP analysis. The septin ring was found to be quite stable
in wild-type cells, as assessed by the turnover of Spn4p–GFP
(F. Chang, personal communication). In contrast, Spn4p–
GFP was considerably more dynamic in mid2Δ cells, indicat-
ing that Mid2p function is required for the normal rigidity of
the septin ring (F. Chang, personal communication).

Other anillin homologues have also been shown to influ-
ence septin ring organization. When C. albicans Int1p is
overproduced in S. cerevisiae, highly ordered ectopic septin
structures are observed in a manner strikingly similar to
when Mid2p is overproduced (Gale et al., 2001). Further,
overproduction of a carboxy-terminal fragment of Hs anillin
induced the formation of ectopic septin containing struc-
tures with which Hs anillin was colocalized (Oegema et al.,
2000). These abnormal cortical foci did not contain either
actin or myosin-II (Oegema et al., 2000), nor did those
formed upon Mid2p overproduction (Fig. 8 A; unpublished
data). Thus, Mid2p shares with these proteins the ability to
interfere with septin organization and function, although it
is currently unknown whether the abnormal septin struc-
tures produced by Mid2p overproduction contain Mid2p.

An outstanding issue is how the events downstream of
septation initiation network activation and septation are
restricted temporally. Because all SCF targets identified to
date are phosphorylated before being recognized by their
cognate F-box protein (Willems et al., 1999), an appealing
hypothesis is that the kinase that presumably modifies
Mid2p in order to initiate its destruction might be condi-
tionally activated upon the completion of septation. The
identification of the protein kinase that phosphorylates
Mid2p would significantly enhance our current under-
standing of the signaling pathways regulating cell separation.

Because Mid2p is only observed at the site of cell division,
it follows then that the destruction machinery must also be
recruited there. A candidate F-box protein is Pof6p, which
was recently shown to localize to each side of the septum in
dividing cells (Hermand et al., 2003). The loss of function
of either Pof6p or the core SCF component Skp1p resulted
in aberrant or multiple septa, implying that elevated levels of certain factors may also inhibit efficient cell division (Herman et al., 2003). Both the loss of the transcription factor Sep1p (Ribar et al., 1999) or a slight increase in its abundance generates a cell separation defect (unpublished data). Thus, the proper coordination of cytokinesis appears to require a fine balance between the activation and inhibition of various factors involved in the process.

The loss of function of a wide array of proteins in S. pombe produces similar cytokinesis defects. For example, deletion of the septins, calcineurin (Ppb1p), a transcription factor (Sep1p), a MAP kinase (Pmk1p), a MAP kinase phosphatase (Pnp1p), members of the exocyst complex (Sec6p), and now Mid2p all cause a long delay in the physical separation of cells after septum synthesis, although the nuclear cycle remains unaffected (J. Pringle, personal communication; Yoshida et al., 1994; Longtine et al., 1996; Toda et al., 1996; Sugiiura et al., 1998; Ribar et al., 1999; Wang et al., 2002). Whereas septin ring disorganization is likely to be the underlying cause of the cell separation defect of mid2Δ cells, this is not the case for ppb1Δ. In ppb1Δ cells, both Spn3p-GFP and Mid2p-GFP localize normally. This result suggests that even though a number of mutants inhibit cell separation in S. pombe, defects in multiple pathways may lead to this phenotype. Determining how septin ring organization is affected in other cytokinesis mutants may help elucidate the pathways regulating cell separation.

Materials and methods

Strains, media, and methods

The S. pombe strains used in this study (Table I) were grown in YE or minimal medium with the appropriate supplements as previously described (Moreno et al., 1990). DNA transformations were done by electroporation (Prentice, 1992) or lithium acetate transformation (Keeney and Boeke, 1994). Induction of the nmt promoter (Basi et al., 1993; Maundrell, 1993) was achieved by growing cells in thiamine (promoter repressed) and then washing cells three times in medium lacking thiamine (promoter induced).

The mid2Δ, spn1Δ, and spn3Δ ORFs were each tagged at their 3′ ends with either the HA3-Kan6, myc13-Kan6, or EGFP-Kan6 cassette, and the spn2 ORF was also tagged with a CFP-Kan6 cassette as previously described (Bähler et al., 1998b). Kan6 transformants were screened by whole cell PCR and then by immunoblotting to confirm the accurate integration and expression of the fusion protein.

The mid2Δ, sepi1Δ, and spn4Δ ORFs were replaced with ura4Δ by homologous recombination in a diploid strain (Bähler et al., 1998b). Ura4 transformants were screened for the proper gene disruptant by whole cell PCR. Heterozygous diploid strains were then sporulated at 25°C followed by tetrad dissection to determine if these genes were essential for viability.

Molecular biology techniques

PCR amplifications were performed with TaqPlus Precision polymerase (Stratagene) according to manufacturer’s instructions. Oligonucleotides

Table I. Strains used in this study

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*University of Massachusetts Medical School, Worcester, MA.
were synthesized by Integrated DNA Technologies, Inc., and all sequences are available upon request. Sequencing was done with Thermosequenase (USB) and Redivue 3’P Terminator Kit (Amersham Biosciences). The entire mid2 ORF was amplified from genomic DNA with oligos containing NdeI and BamHI sites on the 5’ and 3’ ends, respectively. The PCR product was cut with NdeI/BamHI and cloned into the thiamine-repressible pREP1, -41, -41HA, and -41GFP vectors to create pKG2207, pKG2208, pKG2491, and pKG2236. Primers containing 5’ NdeI/ATG and 3’ TAA/BamHI sites were also designed to amplify regions of Mid2p using pKG2208 as a template. These PCR products were also subcloned into the pREP series of vectors.

Cytology and microscopy

Strains producing GFP-tagged proteins were grown in YE medium, visualized live or fixed with ethanol or formaldehyde, and processed as previously described (Balasubramanian et al., 1997; Tomlin et al., 2002). To visualize cell walls, fixed cells were stained with aniline blue (methyl blue) (Sigma-Aldrich). Cells from a 1-ml culture were fixed in ethanol, resuspended in 1 ml of a 1:100 dilution of an aniline blue stock solution (100 mg/ml), incubated for 10 min, collected by centrifugation, washed three times with water, and resuspended in 20-µl water. Lata (Molecular Probes) was used at a concentration of 100 or 200 µM. Actin was visualized after formaldehyde fixation using AlexaFluor®594-phalloidin (Molecular Probes). Images were acquired digitally as previously described (Tomlin et al., 2002). For time-lapse experiments, cells were placed on a hanging drop glass slide (Fisher Scientific) containing solidified YE agar and covered with a coverslip. Time-lapse images of Spn3p–GFP and Mid2p–GFP were obtained using an Ultraview LCI confocal microscope equipped with a 488-nm Ar ion laser (PerkinElmer). Images were captured using Ultraview LCI software (version 5.2; PerkinElmer) and processed using Velocity software (version 1.4.2; Improvision). Z-series optical sections were taken at 0.5-µm spacing at intervals of 2 or 3 min.

Immunoprecipitations and immunoblots

Whole cell lysates were prepared in NP-40 buffer followed by anti-HA or anti-Myc immunoprecipitations as previously described (Gould et al., 1991; McDonald et al., 1999). Denatured lysates were prepared as previously described (Burns et al., 2002). Protein samples were resolved on 4–12% NuPAGE gels in MOPS buffer and subsequently transferred to 0.2 µm nitrocellulose (Bio-Rad Laboratories) according to the manufacturer’s instructions (Invitrogen). Immunoblotting was done with anti-HA (1:25A; 2 µg/ml), anti-Myc (9E10; 2 µg/ml), anti-Cdc2p (PSTAIRE; 1:5,000; Sigma-Aldrich), or anti-ubiquitin (1:100; Sigma-Aldrich). The primary antibodies were detected with HRP-conjugated goat anti–mouse or goat anti–rabbit secondary antibodies (0.4 mg/ml; Jackson ImmunoResearch Laboratories) at a dilution of 1:50,000 followed by ECL visualization using SuperSignal (Pierce Chemical Co.).

Immunoprecipitation/phosphatase assay

After an anti-HA immunoprecipitation from denatured lysate, beads were washed two times with 1 ml NP-40 buffer, four times with 1 ml of phosphatase buffer (25 mM Hapes-NaOH, pH 7.4, 150 mM NaCl, 0.1 mM BSA), divided in half, pulsed down, and the supernatant aspirated off. 10-µl reactions composed of 1X phosphatase buffer, 2 mM MnCl2, plus 1 µl of A-phosphatase (New England Biolabs, Inc.) or 1 µl H2O were then incubated at 30°C for 45 min with gentle mixing every 5 min. The beads were washed three times with NP-40 buffer and resuspended in 25 µl 2X lithium dodecyl sulfate sample buffer.

Northern blotting

Total RNA was prepared by extraction with hot acidic phenol and SDS as detailed previously (Burns et al., 1999). Total RNA (20 µg per sample) was resolved on formaldehyde–agarose gels and capillary blotted to a Duralon-UV membrane. mid2 and his3 mRNA levels were detected by hybridization of 32P-labeled oligonucleotide probes (Rediprime II; Amersham Biosciences) from regions within their ORFs. The blots were exposed to Phosphor-Imager screens and visualized with ImageQuant 5.2 on an Amersham Biosciences Typhoon 9200 scanner.

In vivo ubiquitination assays

Strains carrying pREP1-His6–ubiquitin were grown at 25°C for 22 h in the absence of thiamine to induce the expression of His6–ubiquitin (Ub), shifted to 36°C for 4 h, and then 6 × 106 cells were harvested. Cell lysates were prepared in the presence of 8 M urea with 100 mM sodium phosphate, pH 8.0, and 5 mM imidazole followed by binding of His6–Ub-conjugated proteins to a nickel affinity resin (Ni-NTA Superflow; QIAGEN). The beads were washed as previously described (Benito et al., 1998) and ubiquitinated proteins eluted with 200 mM imidazole.

Online supplemental material

The supplemental material for this article is available at http://www.jcb.org/cgi/content/full/jcb.200211126/DC1. Three Quicktime movie files of the following live cells are available online: Mid2p–GFP (Video 1), Spn3p–GFP (Video 2), and Spn3p–GFP in a mid2Δ strain (Video 3).

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