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Cooperation Between the Septins and the Actomyosin Ring and Role of a Cell-Integrity Pathway During Cell Division in Fission Yeast

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ABSTRACT

A major question about cytokinesis concerns the role of the septin proteins, which localize to the division site in all animal and fungal cells but are essential for cytokinesis only in some cell types. For example, in *Schizosaccharomyces pombe*, four septins localize to the division site, but deletion of the four genes produces only a modest delay in cell separation. To ask if the *S. pombe* septins function redundantly in cytokinesis, we conducted a synthetic-lethal screen in a septin-deficient strain and identified seven mutations. One mutation affects Cdc4, a myosin light chain that is an essential component of the cytokinetic actomyosin ring. Five others cause frequent cell lysis during cell separation and map to two loci. These mutations and their dosage suppressors define a signaling pathway (including Rho1 and a novel arrestin) for repairing cell-wall damage. The seventh mutation affects the poorly understood RNA-binding protein Scw1 and severely delays cell separation when combined either with a septin mutation or with a mutation affecting the septin-interacting, anillin-like protein Mid2, suggesting that Scw1 functions in a pathway parallel to that of the septins. Taken together, our results suggest that the *S. pombe* septins participate redundantly in one or more pathways that cooperate with the actomyosin ring during cytokinesis and that a septin defect causes septum defects that can be repaired effectively only when the cell-integrity pathway is intact.

THE fission yeast *Schizosaccharomyces pombe* provides an outstanding model system for studies of cytokinesis (McCollum and Gould 2001; Balasubramanian *et al.* 2004; Pollard and Wu 2010). As in most animal cells, successful cytokinesis in *S. pombe* requires an actomyosin ring (AMR). The AMR begins to assemble at the G_2/M transition and involves the type II myosin heavy chains Myo2 and Myp2 and the light chains Cdc4 and Rlc1 (Wu *et al.* 2003). Myo2 and Cdc4 are essential for cytokinesis under all known conditions, Rlc1 is important at all temperatures but essential only at low temperatures, and Myp2 is essential only under stress conditions. As the AMR constricts, a septum of cell wall is formed between the daughter cells. The primary septum is sandwiched by secondary septa and subsequently digested to allow cell separation (Humbel *et al.* 2001; Sipiczki 2007). Because of the internal turgor pressure of the cells, the proper assembly and structural integrity of the septal layers are essential for cell survival.

Septum formation involves the β-glucan synthases Bgs1/Cps1/Drc1, Bgs3, and Bgs4 (Ishiguro *et al.* 1997; Le Goff *et al.* 1999; Liu *et al.* 1999, 2002; Martín *et al.* 2003; Cortés *et al.* 2005) and the α-glucan synthase Ags1/Mok1 (Hochstenbach *et al.* 1998; Katayama *et al.* 1999). These synthases are regulated by the Rho GTPases Rho1 and Rho2 and the protein kinase C isoforms Pck1 and Pck2 (Arellano *et al.* 1996, 1997, 1999; Nakano *et al.* 1997; Hirata *et al.* 1998; Calonge *et al.* 2000; Sayers *et al.* 2000; Ma *et al.* 2006; Barba *et al.* 2008; García *et al.* 2009b). The Rho GTPases themselves appear to be regulated by both GTPase-activating proteins (GAPs) and guanine-nucleotide-exchange factors (GEFs) (Nakano *et al.* 2001; Calonge *et al.* 2003; Iwaki *et al.* 2003; Tajadura *et al.* 2004; Morrell-Falvey *et al.* 2005; Mutoh *et al.* 2005; García *et al.* 2006, 2009a,b). In addition, septum formation and AMR function appear to be interdependent. In the absence of a normal AMR, cells form aberrant septa and/or deposit septal materials at random locations, whereas a mutant defective in septum formation (*bgs1*) is also

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defective in AMR constriction (Gould and Simanis 1997; Le Goff *et al.* 1999; Liu *et al.* 1999, 2000). Both AMR constriction and septum formation also depend on the septation initiation network involving the small GTPase Spg1 (McCollum and Gould 2001; Krapp and Simanis 2008). Despite this considerable progress, many questions remain about the mechanisms and regulation of septum formation and its relationships to the function of the AMR.

One major question concerns the role(s) of the septins. Proteins of this family are ubiquitous in fungal and animal cells and typically localize to the cell cortex, where they appear to serve as scaffolds and diffusion barriers for other proteins that participate in a wide variety of cellular processes (Longtine *et al.* 1996; Gladfelter *et al.* 2001; Hall *et al.* 2008; Caudron and Barral 2009). Despite the recent progress in elucidating the mechanisms of septin assembly (John *et al.* 2007; Sirajuddin *et al.* 2007; Bertin *et al.* 2008; McMurray and Thorner 2008), the details of septin function remain obscure. However, one prominent role of the septins and associated proteins is in cytokinesis. Septins concentrate at the division site in every cell type that has been examined, and in *Saccharomyces cerevisiae* (Hartwell 1971; Longtine *et al.* 1996; Lippincott *et al.* 2001; Dobbelaere and Barral 2004) and at least some Drosophila (Neufeld and Rubin 1994; Adam *et al.* 2000) and mammalian (Kinoshita *et al.* 1997; Surka *et al.* 2002) cell types, the septins are essential for cytokinesis. In *S. cerevisiae*, the septins are required for formation of the AMR (Bi *et al.* 1998; Lippincott and Li 1998). However, this cannot be their only role, because the AMR itself is not essential for cytokinesis in this organism (Bi *et al.* 1998; Korinek *et al.* 2000; Schmidt *et al.* 2002). Moreover, there is no evidence that the septins are necessary for AMR formation or function in any other organism. A further complication is that in some cell types, including most *Caenorhabditis elegans* cells (Nguyen *et al.* 2000; Maddox *et al.* 2007) and some Drosophila cells (Adam *et al.* 2000; Field *et al.* 2008), the septins do not appear to be essential for cytokinesis even though they localize to the division site.

S. pombe has seven septins, four of which (Spn1, Spn2, Spn3, and Spn4) are expressed in vegetative cells and localize to the division site shortly before AMR constriction and septum formation (Longtine *et al.* 1996; Berlin *et al.* 2003; Tasto *et al.* 2003; Wu *et al.* 2003; An *et al.* 2004; Petit *et al.* 2005; Pan *et al.* 2007; Onishi *et al.* 2010). Spn1 and Spn4 appear to be the core members of the septin complex (An *et al.* 2004; McMurray and Thorner 2008), and mutants lacking either of these proteins do not assemble the others at the division site. Assembly of a normal septin ring also depends on the anillin-like protein Mid2, which colocalizes with the septins (Berlin *et al.* 2003; Tasto *et al.* 2003). Surprisingly, mutants lacking the septins are viable and form seemingly complete septa with approximately normal timing. These mutants do, however, display a variable delay in separation of the daughter cells, suggesting that the septins play some role(s) in the proper completion of the septum or in subsequent processes necessary for cell separation (Longtine *et al.* 1996; An *et al.* 2004; Martín-Cuadrado *et al.* 2005).

It is possible that the septins localize to the division site and yet are nonessential for division in some cell types because their role is redundant with that of some other protein(s) or pathway(s). To explore this possibility in *S. pombe*, we screened for mutations that were lethal in combination with a lack of septins. The results suggest that the septins cooperate with the AMR during cytokinesis and that, in the absence of septin function, the septum is not formed properly, so that an intact system for recognizing and repairing cell-wall damage becomes critical for cell survival.

MATERIALS AND METHODS

Strains, plasmids, growth conditions, and genetic methods: *S. pombe* strains are listed in Table 1 and/or described below; all are congenic to strain 972 (Leupold 1970). Plasmid pREP42::GFP-cdc4 was described by Balasubramanian *et al.* (1997). Plasmids with overlapping inserts covering nucleotides 627,249–681,978 of chromosome III were provided by the Yeast Genetic Resource Center Japan (YGRC/NBRP; http://yeast.lab.nig.ac.jp/nig/). Three *S. pombe* genomic DNA libraries were used: one with large inserts (provided by P. Young, Queens University, Ontario, Canada), constructed in plasmid pWH5 (Wright *et al.* 1986), and two with smaller inserts (pURSP1 and pURSP2, provided by A. Carr, University of Sussex, Falmer, UK), constructed in plasmid pUR19.

Standard rich (YE5S) and minimal (EMM) media and genetic methods were used (Moreno *et al.* 1991); EMM5S medium was EMM containing also adenine, histidine, leucine, lysine, and uracil. To repress expression from the *nmt1* promoter, 5 μg/ml thiamine was added to the medium. Some solid media contained 2.5 μg/ml phloxin B, which accumulates in dead cells to give a red colony color (Moreno *et al.* 1991). Standard molecular biology methods were used except where noted. Oligonucleotide primers were purchased from Integrated DNA Technologies.

Mutagenesis and screening for synthetic-effect mutations: To regulate the expression levels of *spn1*$^+$ and *spn4*$^+$, their promoters were replaced with the attenuated *81nmt1* promoter in wild-type strain 972 by the PCR method (Bähler *et al.* 1998) using the *kanMX6* marker, forward primers corresponding to nucleotides −111 to −42 (*spn1*) or −134 to −55 (*spn4*) relative to the start codons, and reverse primers based on the N-terminal codons. In both cases, the upstream ORFs are far enough away that these manipulations are unlikely to affect their expression. Checking by PCR identified transformants (JW254 and JW188) that had sustained the desired integrations, and G418 resistance segregated 2:2 in crosses of these strains to JW215. Appropriate segregants were then crossed to obtain strain JW267 (*81nmt1-spn1*$^+$ *81nmt1-spn4*$^+$).

To screen for synthetic-effect mutations, strain JW267 was grown in EMM5S to ~10^7 cells/ml, mutagenized with 300 μg/ml nitrosoguanidine (Sigma-Aldrich) at 30° for 45 min (~20% survival) as described by Moreno *et al.* (1991), and plated onto EMM5S (~500 viable cells/plate) at 30°. The resulting colonies were replica-plated onto EMM5S + phloxin (inducing conditions) and EMM5S + thiamine + phloxin (repressing conditions) at 30°. Colonies that were significantly redder or

TABLE 1

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

Strain	Genotype[a]	Source/reference
972	*h*⁻ wild type	Leupold (1970)
DM1274	*h*⁻ *scw1Δ::ura4*⁺ *ade6-M210 leu1-32 ura4-D18*	Jin and McCollum (2003)
FY435	*h*⁺ *ade6-M210 his7-366 leu1-32 ura4-D18*	S. Forsburg
FY11065	*h*⁻ *cds1-2HA-6His::ura4*⁺ *leu1 ura4*	Yeast Genetic Resource Center Japan
KS3320	*h*⁺ *swi5Δ::kanMX6*	Anders *et al.* (2008)
KS3250	*h*⁻ *rec12Δ::kanMX6 ade6-M210 his7-366 leu1-32 lys1-37*	Anders *et al.* (2008)
MBY580	*h*⁻ *bgs1-191 ade6-M210 leu1-32 lys1-131 ura4-D18*	Liu *et al.* (1999)[b]
TP5	*h*⁻ *myp2Δ::his7*⁺ *ade6-M210 his7-366 leu1-32 ura4-D18*	Bezanilla *et al.* (1997)
TP90	*h*⁺ *myo2-E1 myp2-Δ1::his7*⁺ *ade6-M216 his7-366 leu1-32 ura4-D18*	M. Bezanilla and T. Pollard
YDM74	*h*⁻ *myo2-E1 ade6 his3-D1 leu1-32 ura4-D18*	D. McCollum[c]
JW21	*h*⁺ *cdc4-8*	P. Nurse; Nurse *et al.* (1976); McCollum *et al.* (1995)
JW81	*h*⁻ *ade6-M210 leu1-32 ura4-D18*	Lab collection
JW182	*h*⁻ *spn4-Δ2*	This study[d]
JW183	*h*⁻ *spn4*⁺*-GFP*	This study[d]
JW215	*h*⁺ *his3-27 leu1-32 ura4-D18*	Lab collection
JW249	*h*⁻ *spn1-Δ2*	This study[d]
JW251	*h*⁻ *spn1*⁺*-GFP*	This study[d]
JW267	*h*⁻ *81nmt1-spn1*⁺ *81nmt1-spn4*⁺ *leu1-32 ura4-D18*	See text.
JW289	*h*⁺ *spn1-Δ2 leu1-32 ura4-D18*	JW215 × JW249
JW290	*h*⁻ *spn1-Δ2 his3-27 ura4-D18*	JW215 × JW249
JW295	*h*⁺ *spn4-Δ2 leu1-32 ura4-D18*	JW215 × JW182
JW306	*h*⁺ *spn1*⁺*-GFP his3-27 leu1-32 ura4-D18*	JW215 × JW251
JW314	*spn4-Δ2 mid2Δ::ura4*⁺ *leu1-32 ura4-D18*	JW295 × JW430
JW315	*scw1-ng124 mid2Δ::ura4*⁺ *leu1-32 ura4-D18*	*ng124*[e] × JW430
JW318	*spn1*⁺*-GFP mid2Δ::ura4*⁺ *leu1-32 ura4-D18*	JW306 × JW430
JW320	*scw1-ng124 spn4*⁺*-GFP leu1-32 ura4-D18*	JW331 × JW183
JW321-1	*scw1-ng124 spn1-Δ2 ura4-D18*	JW331 × JW290
JW326	*h*⁻ *mid2*⁺*-GFP ade6-M210 leu1-32 ura4-D18*	See text.
JW330	*h*⁻ *scw1-ng124 ade6-M210 leu1-32 ura4-D18*	This study[f]
JW331	*h*⁺ *scw1-ng124 ade6-M210 leu1-32 ura4-D18*	This study[f]
JW332	*mid2*⁺*-GFP spn4-Δ2 leu1-32 ura4-D18*	JW326 × JW295
JW345	*cdc4-s16 myp2Δ::his7*⁺ *ade6-M210 his7-366 leu1-32 ura4-D18*	JW400 × TP5
JW374	*art1-s34 spn1-Δ2 leu1-32 ura4-D18*	JW403 × JW289
JW380	*h*⁻ *myp2Δ::his7*⁺ *spn1-Δ2 ade6 his7-366 leu1-32 ura4-D18*	This study[g]
JW396	*h*⁻ *s26 leu1-32 ura4-D18*	This study[h]
JW397	*h*⁻ *s63 his3-27 leu1-32 ura4-D18*	This study[h]
JW398	*h*⁺ *s28 leu1-32 ura4-D18*	This study[h]
JW399	*h*⁻ *cdc4-s16 leu1-32 ura4-D18*	This study[e]
JW400	*h*⁺ *cdc4-s16 ade6-M210 his7-366 leu1-32 ura4-D18*	JW399 × FY435
JW401	*cdc4-s16 spn4-Δ2 leu1-32 ura4-D18*	JW399 × JW295
JW402	*h*⁻ *art1-s34 81nmt1-spn1*⁺ *81nmt1-spn4*⁺ *leu1-32 ura4-D18*	This study[i]
JW403	*h*⁻ *art1-s34 leu1-32 ura4-D18*	This study[e]
JW404	*h*⁺ *art1-s34 leu1-32 ura4-D18*	This study[e]
JW405	*h*⁻ *rgf3-s44 81nmt1-spn1*⁺ *81nmt1-spn4*⁺ *leu1-32 ura4-D18*	This study[i]
JW406	*h*⁻ *rgf3-s44 his3-27 leu1-32 ura4-D18*	This study[e]
JW407	*h*⁺ *rgf3-s44 his3-27 leu1-32 ura4-D18*	This study[e]
JW409	*h*⁻ *rgf1-GFP ade6-M210 leu1-32 ura4-D18*	See text.
JW412	*h*⁻ *rgf3-GFP ade6-M210 leu1-32 ura4-D18*	See text.
JW423	*h*⁻ *rgf1-Δ1 ade6 leu1-32 ura4-D18*	This study[j]
JW430	*h*⁻ *mid2Δ::ura4*⁺ *ade6 leu1-32 ura4-D18*	D. Brunner and P. Nurse
JW729	*h*⁺ *ade6-M210 leu1-32 ura4-D18*	Lab collection
JW903	*h*⁺ *3nmt1-rgf2 ade6-M210 leu1-32 ura4-D18*	See text.
JW1078	*h*⁻ *3nmt1-rgf1 ade6-M210 leu1-32 ura4-D18*	See text.
JW1081	*h*⁻ *3nmt1-rgf3 ade6-M210 leu1-32 ura4-D18*	See text.
JW1084	*h*⁻ *sad1-mRFP1 ade6-M210 leu1-32 ura4-D18*	See text.
JW1100	*h*⁺ *spn1-mEGFP ade6-M210 leu1-32 ura4-D18*	See text.
JW1105	*h*⁺ *rgf3-mEGFP ade6-M210 leu1-32 ura4-D18*	See text.

(*continued*)

TABLE 1

(Continued)

Strain	Genotype[a]	Source/reference
JW1113	*h⁻ spn1-mEGFP sad1-mRFP1 ade6-M210 leu1-32 ura4-D18*	JW1100 × JW1084
JW1122	*3nmt1-rgf3 spn1-mEGFP ade6-M210 leu1-32 ura4-D18*	JW1081 × JW1100
JW1123	*3nmt1-rgf1 spn1-mEGFP ade6-M210 leu1-32 ura4-D18*	JW1078 × JW1100
JW1124	*rgf1-GFP sad1-mRFP1 ade6-M210 leu1-32 ura4-D18*	This study[k]
JW1125	*rgf3-s44 spn1-mEGFP leu1-32 ura4-D18*	JW406 × JW1100
JW1126	*art1-s34 spn1-mEGFP ade6-M210 leu1-32 ura4-D18*	JW403 × JW1100
JW1128	*rgf3-GFP spn1-Δ2 ade6-M210 leu1-32 ura4-D18*	JW412 × JW289
JW1131	*rgf3-mEGFP sad1-mRFP1 ade6-M210 leu1-32 ura4-D18*	JW1105 × JW1084
JW1139	*rgf1-GFP spn1-Δ2 ade6-M210 leu1-32 ura4-D18*	JW409 × JW289
JW1203	*mid2-GFP scw1-ng124 ade6-M210 leu1-32 ura4-D18*	JW326 × JW331
JW2068	*h⁺ sec15Δ::kanMX4 ade6 leu1-32 ura4-D18*	Lab collection
JW2155	*h⁻ scw1-ng124 ade6-M210 leu1-32 ura4-D18*	This study[l]
JW2271	*h⁺ scw1-ng124 ade6-M216 leu1-32 ura4-D18*	This study[l]
JW2286	*scw1Δ::ura4⁺ spn1-Δ2 ade6-M210 leu1-32 ura4-D18*	DM1274 × JW289
JW2499	*cdc4-s16 scw1-ng124 ade6 leu1-32 ura4-D18*	JW399 × JW2271
JW2503	*myo2-E1 scw1-ng124 ade6 leu1-32 ura4-D18*	YDM74 × JW2271
JW2560	*myo2-E1 myp2-Δ1::his7⁺ spn1-Δ2 ade6 his7-366 leu1-32 ura4-D18*	This study[m]
JW2613	*h⁺/h⁻ scw1-ng124/scw1⁺ ade6-M216/ade6-M210 leu1-32/leu1-32 ura4-D18/ura4-D18*	JW2271 × JW81
JW2614	*h⁺/h⁻ scw1-ng124/scw1Δ::ura4⁺ ade6-M216/ade6-M210 leu1-32/leu1-32 ura4-D18/ura4-D18*	DM1274 × JW2271

[a] All deletions and taggings constructed in this study by the PCR method used the *kanMX6* marker, which is not indicated in the genotypes. The mating types of some strains were not determined. *GFP(S65T)* is indicated simply as *GFP*; *mEGFP* (see MATERIALS AND METHODS) is indicated as such.

[b] *bgs1-191* was originally known as *drc1-191* (LIU *et al.* 1999).

[c] *myo2-E1* was originally referred to as *rng5-E1* (BALASUBRAMANIAN *et al.* 1998; ENG *et al.* 1998).

[d] Strain 972 was transformed with the appropriate constructs (see text).

[e] Segregant from the third backcross of the original mutant isolate to strains JW81 and JW215.

[f] Segregant from the fourth backcross of the original mutant isolate to strains JW81 and JW215.

[g] Derived by several crosses from strains TP5 and JW249.

[h] Segregant from the first backcross of the original mutant isolate to strain JW215.

[i] The original mutant isolate.

[j] Segregant from a heterozygous mutant obtained after PCR-mediated transformation (see text) of diploid strain JW9 (WU *et al.* 2001).

[k] Derived by crossing JW409 to a segregant from the cross of JW1084 to JW729 (WU *et al.* 2003).

[l] Segregant from the 10th backcross of the original mutant isolate to strains JW81 and JW729.

[m] Derived by several crosses from strains TP5, YDM74, and JW249.

grew significantly less well on the thiamine plates were restreaked under inducing and repressing conditions. Possible mutants with modest growth defects were also screened microscopically to identify those with strong morphological phenotypes. Before detailed characterization, each mutant was backcrossed three or more times to wild-type strains JW81, JW215, and/or JW729 to obtain strains that carried the new mutations without *81nmt1-spn1* or *81nmt1-spn4*.

Identification of the genes defined by synthetic-effect mutations: To clone the gene defined by mutation *s16*, *spn4*Δ *s16* strain JW401 was transformed with the pWH5 and pURSP2 libraries, grown at 36° on EMM5S-Leu or EMM5S-Ura, and replica-plated onto phloxin-containing plates at 23°. Three transformants with reduced phloxin uptake were found. After recovery in *Escherichia coli*, all three plasmids could rescue the mutant phenotypes of strains JW401 and JW399 (*s16*) to *spn4*Δ-like and wild-type phenotypes, respectively, at 23°. Sequencing revealed that the plasmids all contained the complete *cdc4* coding sequence and the C-terminal ~190 codons of the adjacent, 590-codon *paa1*. To ask if *s16* was a mutation in *cdc4*, a genomic DNA fragment that extended from nucleotide −197 upstream to nucleotide 173 downstream of the *cdc4* coding region was amplified from strain JW399 using the Expand High Fidelity PCR system (Roche). The PCR product was purified using Qiagen columns and sequenced.

To clone the genes defined by mutations *s34* and *s44*, strains JW402 (*s34 81nmt1-spn1⁺ 81nmt1-spn4⁺*) and JW405 (*s44 81nmt1-spn1⁺ 81nmt1-spn4⁺*) were grown in EMM5S medium, transformed with the pURSP1 and pURSP2 libraries, and plated on EMM5S-Ura + thiamine at 30°. Plasmids from large colonies were recovered into *E. coli*, and those that could at least partially rescue the growth defects and cell-lysis phenotypes of the mutant strains upon retransformation were classified by restriction digestion and partial sequencing (see RESULTS). To identify the site of the *s34* mutation, genomic DNA fragments from strain JW404 were amplified using the Expand or iProof (Bio-Rad) high-fidelity PCR system, purified on Qiagen columns, and sequenced. Collectively, the fragments covered 25,089 bp around *bgs1* on chromosome II (nucleotides 2,341,116–2,366,204). The same approach was used to identify the *s44* mutation in strain JW407. The region sequenced covered 10,321 bp (nucleotides 1,239,244–1,249,564) on chromosome III, which includes the entire ORFs of the adjacent and divergently transcribed *rgf1* and *rgf3*, the 2050-bp region

between them, and 141 and 251 bp of their respective 3′ UTRs. In both cases, the mutant sites were confirmed by sequencing several fragments from independent PCR reactions.

To identify the gene defined by mutation *ng124*, we used positional cloning. First, we used strains containing a *rec12*Δ mutation [which reduces homologous recombination by ~1000-fold when homozygous (Anders *et al.* 2008)] and markers on each chromosome. This revealed cosegregation of *ng124* with *ade6*$^+$, indicating that *ng124* is on chromosome III. Next, we used strains carrying *swi5*Δ [which reduces recombination by ~10-fold when homozygous (Schmidt 1993)], revealing linkage of *ng124* to *ura4* (~15 cM in the *swi5*Δ background). Crosses using *swi5*$^+$ strains then showed that *ng124* was ~7 cM from *sec15* and ~12 cM from *cds1*, suggesting that *ng124* lies between these two genes (which are ~20 cM apart). One gene in this region is *scw1*, mutations in which have been shown also to produce a multi-septum phenotype (Karagiannis *et al.* 2002; Jin and McCollum 2003). Several lines of evidence suggested that *ng124* is indeed a mutation in *scw1* (see results), and this was confirmed by sequencing genomic DNA (chromosome III nucleotides 674,476–675,161) from an extensively backcrossed *ng124* strain (JW2155), which revealed a mutation in *scw1* codon 276 (see results).

Gene deletion and tagging: Genes were deleted (from start codon to stop codon) or tagged at the C termini of their chromosomal loci by the PCR method (Bähler *et al.* 1998; Wu *et al.* 2001) using the *kanMX6* marker. The tags encoded GFP (green fluorescent protein with the S65T mutation), mEGFP [GFP with the F64L and S65T mutations to enhance fluorescence and the A206K mutation to minimize dimerization (Yang *et al.* 1996; Zacharias *et al.* 2002; Wu *et al.* 2003)], or mRFP1 [monomeric red fluorescent protein (Campbell *et al.* 2002; Huh *et al.* 2003)]. The gene-specific sequences of the forward primers ended immediately upstream of the stop codons, and those of the reverse primers ended short distances downstream of the stop codons (1 bp for *rgf1*, 10 bp for *rgf2*, 8 bp for *rgf3*, 28 bp for *spn1*, 44 bp for *spn4*, 5 bp for *mid2*, and 1 bp for *sad1*). All of the tagged proteins appeared to be functional as judged by normal colony growth and normal cell morphologies in the tagged strains (Wu *et al.* 2003; data not shown). The PCR method was also used to place genes under the control of the strong (*3nmt1*) *nmt1* promoter (Basi *et al.* 1993; Forsburg 1993); the gene-specific sequences of the forward primers ended short distances upstream of the start codons (5 bp for *rgf1* and 6 bp for *rgf2* and *rgf3*) and those of the reverse primers ended immediately downstream of the start codons. Transformants were recovered on YE5S + thiamine to repress the *nmt1* promoter.

Morphological observations: In most cases, cells were observed by fluorescence or differential-interference-contrast (DIC) microscopy using either a Nikon Eclipse 600 FN microscope with a Plan-Apo 100X/1.4 NA objective and a Hamamatsu ORCA-2 cooled CCD camera or an Olympus IX-71 inverted microscope with a Plan-Apo 60X/1.4 NA objective and an ORCA-ER cooled CCD camera. The three-dimensional images in Figure 6 were obtained using an UltraView RS spinning-disk confocal microscope (Perkin Elmer Life Sciences) and a 488-nm argon ion laser. Septa and DNA were stained using Calcofluor and bisbenzamide as described by Wu *et al.* (2001). For time-lapse experiments, cells were pre-grown in the appropriate medium to ~4×10^6 cells/ml, mounted in growth chambers prepared as described by Maddox *et al.* (2000) and Wu *et al.* (2003), and observed at 24° or 25°. Images were collected at 30-sec to 2-min intervals using exposures of 0.065–0.3 sec (DIC) or 0.1–3 sec (GFP) and analyzed using MetaMorph (Molecular Devices) or Image J (http://rsb.info.nih.gov/ij/) software.

RESULTS

Identification of synthetic-effect mutations: To identify proteins or pathways that might be redundant with the septins, we sought mutations that were lethal (or nearly so) when the septins were absent. We used a strain in which both *spn1*$^+$ and *spn4*$^+$ are under control of the weak, thiamine-repressible *81nmt1* promoter. Under inducing conditions, these cells resembled wild type, but under repressing conditions, they resembled *spn1* and *spn4* deletion mutants: the growth rate was close to that of wild type, but many more cells than normal had visible septa (and sometimes several septa), indicating a delay in septum completion and/or cell separation (Figure 1, A–D). We mutagenized with nitrosoguanidine and screened ~150,000 colonies, obtaining seven mutants that died or grew poorly under repressing conditions (see materials and methods). Strains containing only the new mutations were isolated by backcrossing to wild type, and all had defects detectable by phloxin staining, by microscopic examination, or both (see below). Each mutant segregated 2:2 for this phenotype, indicating that a single mutation was involved. Data presented below show that mutation *s16* is in *cdc4*. Linkage analyses (Table 2, lines 1–3 and 5–13) indicated that mutations *s34*, *s26*, and *s63* are tightly linked to each other and therefore likely to define a second gene; that mutations *s44* and *s28* are tightly linked to each other, but not to *s34*, *s26*, and *s63*, and therefore likely to define a third gene; and that mutation *ng124* defines a fourth gene that is not linked to any of the other three.

Genetic interaction between the septins and Cdc4: The *s16 81nmt1-spn1*$^+$ *81nmt1-spn4*$^+$ triple mutant was viable at temperatures ranging from 23 to 36°. It seemed possible that the synthetic phenotype was ameliorated by residual septin expression from the *nmt1* promoter under repressing conditions. Indeed, when *s16* single-mutant (JW399) and *spn4*Δ (JW295) strains were crossed, 23 of 23 predicted double mutants were inviable and arrested as one or several highly elongated cells when tetrads were dissected on YE5S plates at 25° (data not shown). At 32°, 18 of 23 predicted double mutants were viable. Each inviable segregant arrested as a single, highly elongated cell, and most cells of a viable strain (JW401) had detectable defects in cytokinesis and/or septation. At 23°, although both single mutants grew at nearly normal rates, the double mutant grew very slowly (Figure 1E), and although some cells of the *s16* single mutant formed partial or aberrant-looking septa (Figure 1I), the phenotype of the double mutant was much more severe: nearly all cells were highly elongated and had no or aberrant septa, and some cells were lysed (Figure 1M).

To identify the gene containing *s16*, we isolated several complementing plasmids, all of which contained *cdc4* (see materials and methods). Moreover, when strains JW399 (*s16*) and JW401 (*s16 spn4*Δ) were trans-

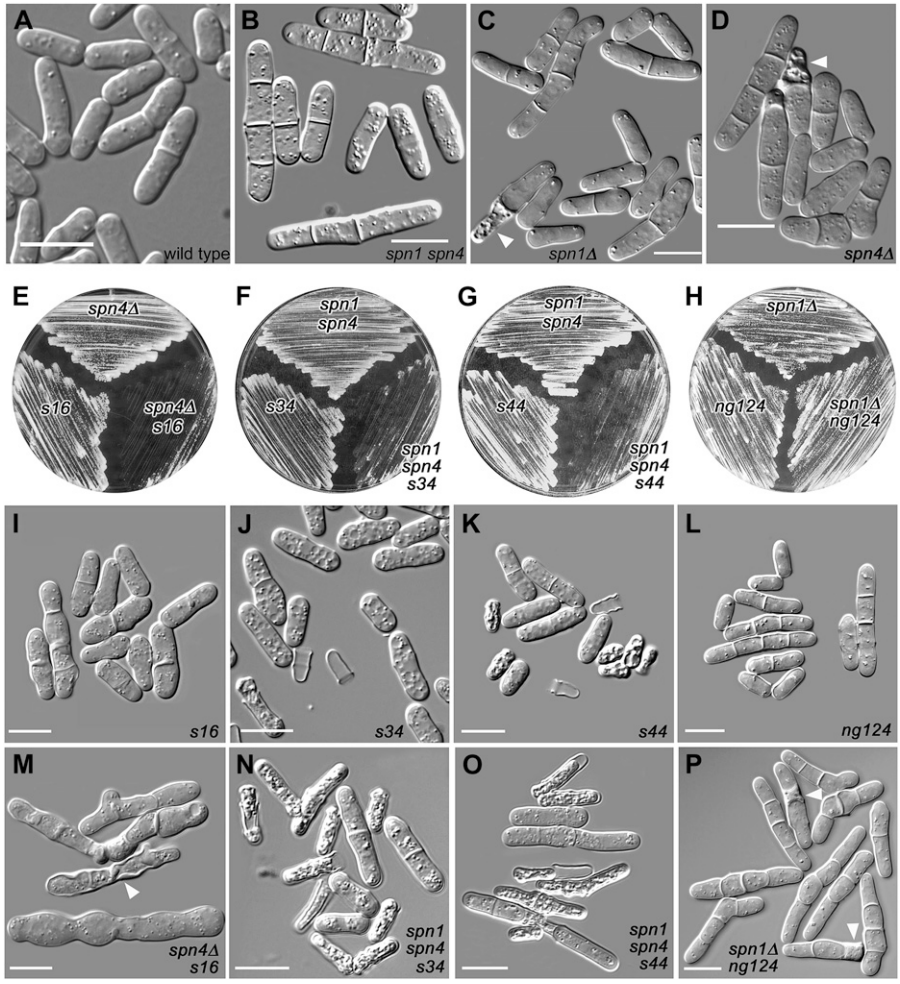

FIGURE 1.—Mutations showing synthetic effects with septin mutations. Cells were observed directly in liquid culture medium by DIC microscopy (A–D and I–P) or streaked on plates to observe overall growth rates (E–H). (A–D) Wild-type strain JW81 and the septin-mutant strains JW267, JW289, and JW182. (I–L) Strains carrying the new mutations in otherwise wild-type backgrounds (JW399, JW403, JW406, and JW330). (M–P) The double or triple mutants (JW401, JW402, JW405, and JW321-1). Plates show the same strains. *spn1 spn4* designates *81nmt1-spn1*$^{+}$ *81nmt1-spn4*$^{+}$. (B, J, K, N, and O) Cells were grown in EMM5S without thiamine and then shifted to EMM5S + thiamine at 30° for 24 hr to repress the *nmt1* promoters. (A, C, L, and P) Cells were grown in YE5S at 30°. (D, I, and M) Cells were grown overnight in EMM5S at 32° and then shifted to 23° for 8 hr. (E) Cells were grown on YE5S at 23° for 5 days. (F and G) Cells were grown on EMM5S + thiamine at 36° for 3 days. (H) Cells were grown on YE5S at 30° for 2 days. Arrowheads in C, D, M, and P indicate cells that appear to have lysed; such cells are numerous in J, K, N, and O. Bars, 10 μm.

formed with plasmid pREP42::GFP-cdc4, which expresses GFP-tagged Cdc4p from the medium-strength *41nmt1* promoter, they displayed wild-type and *spn4*Δ-like phenotypes, respectively, under inducing conditions (data not shown). In addition, *s16* and the temperature-sensitive *cdc4-8* allele are tightly linked. When JW399 was crossed to strain JW21 (*cdc4-8*), no wild-type segregants were detected after dissecting 19 tetrads (incubating segregants at 30°) and analyzing 468 random spores (half germinated at 32° and half at 25°). Finally, sequencing *cdc4* from an *s16* strain (see MATERIALS AND METHODS) revealed a single G-to-A substitution in codon 36 that would result in the replacement of a glycine (conserved among myosin light chains) with glutamic acid. Because *cdc4* is an essential gene (MCCOLLUM *et al.* 1995; NAQVI *et al.* 1999), the *cdc4-s16* allele is presumably a hypomorphic or neomorphic allele that causes only a partial loss of Cdc4 function.

Among other binding partners (see DISCUSSION), Cdc4 is thought to be a common light chain for both type II myosin heavy chains, Myo2 and Myp2. Consistent with this model, we observed strong synthetic phenotypes between *cdc4-s16* and both *myp2* and *myo2* mutations. When *cdc4-s16* and *myp2*Δ strains were crossed, eight of nine predicted double mutants died or grew very poorly (Figure 2A), and only one grew well enough to culture further. At 30°, the single mutants had only mild cell-division defects (Figure 2, B and C), but the double mutant was severely defective in cytokinesis and septation (Figure 2D). Similarly, when strains JW400 (*cdc4-s16*) and YDM74 (*myo2-E1*, a temperature-sensitive allele) were crossed, all nine predicted double mutants from tetrads dissected on YE5S medium at 25° died as highly elongated cells (data not shown).

The septins localize to the division site ~10 min before the initiation of septum formation (BERLIN *et al.* 2003; TASTO *et al.* 2003; WU *et al.* 2003), which is considerably later than Myo2 but similar to Myp2 (KITAYAMA *et al.* 1997; BEZANILLA *et al.* 2000; WU *et al.* 2003). Thus, it seemed possible that the septins might play a role in Myp2 localization or function; if so, then the synthetic lethality between *cdc4-s16* and septin mutations might have the same basis as that between *cdc4-s16* and *myp2*Δ, namely a partial loss of Myo2 function (due to the *cdc4-s16* mutation) that would make full Myp2 function essential. To investigate this possibility, we used *myo2-E1* to reduce Myo2 function and generated *myo2-E1 spn*Δ double mutants by crossing

TABLE 2

Linkage analysis of mutations identified in the synthetic-effect screen

Line	Cross	Tetrad type PD	NPD	T
1	*s34* × *s26*	41	0	0
2	*s34* × *s63*	43	0	0
3	*s44* × *s28*	37	0	0
4	*s28* × *rgf1-Δ1*[a]	20	0	0
5	*s34* × *s28*	1	5	4
6	*s34* × *s44*	1	0	6
7	*s26* × *s28*	2	0	7
8	*s63* × *s28*	1	1	8
9	*ng124* × *s16*	2	0	7
10	*ng124* × *s26*	2	1	7
11	*ng124* × *s34*	0	0	3
12	*ng124* × *s63*	1	4	8
13	*ng124* × *s44*	2	0	5

Appropriate single-mutant strains (Table 1) were crossed, and tetrads were dissected and incubated at 30° or 32°. For crosses involving *s44*, spores were germinated on YE5S + 1 M sorbitol to reduce cell lysis. Mutant and wild-type segregants were distinguished by growing on YE5S medium containing phloxin B or (for *ng124*) by checking the morphological phenotypes microscopically.

[a] Strain JW423 (Table 1).

strain YDM74 to JW289 and JW295. However, although *myo2-E1* and *myp2*Δ show a synthetic phenotype at all temperatures tested and are synthetically lethal at 34–36° (Bezanilla and Pollard 2000; Motegi *et al.* 2000), the *myo2-E1 spn*Δ strains displayed no obvious synthetic phenotypes at 25–36°. In addition, Myp2 localization appeared normal in an *spn1*Δ strain (Wu *et al.* 2003). Similarly, *myp2*Δ *spn1*Δ double mutants (from a cross of strains TP5 and JW289) displayed no obvious synthetic phenotypes at 18–36°, suggesting that the lack of septins does not significantly compromise the function of Myo2. Even a *myo2-E1 myp2*Δ *spn1*Δ triple mutant showed no synthetic growth defect at 23–32° (Figure 2E), and the severity of its cytokinesis defect was similar to that of the *myo2-E1 myp2*Δ double mutant (Figure 2F). Finally, although a wide variety of mutations with mild effects on the AMR become lethal when combined with a *clp1*Δ protein–phosphatase mutation (Mishra *et al.* 2004), an *spn1Δ clp1*Δ double mutant displayed no detectable synthetic effect at 18–36° (data not shown). Thus, the synthetic lethality between *cdc4-s16* and *spn1*Δ or *spn4*Δ does not appear to result from synergistic effects of the *cdc4* and *spn* mutations on the function of the AMR and presumably has some other basis (see Discussion).

Interaction between the septins and a cell-wall-integrity pathway: As described above, mutations *s34*, *s26*, and *s63* mapped to one locus and *s44* and *s28* to another. These mutants all had a similar phenotype that was strongest in *s34* and *s44*, which were therefore

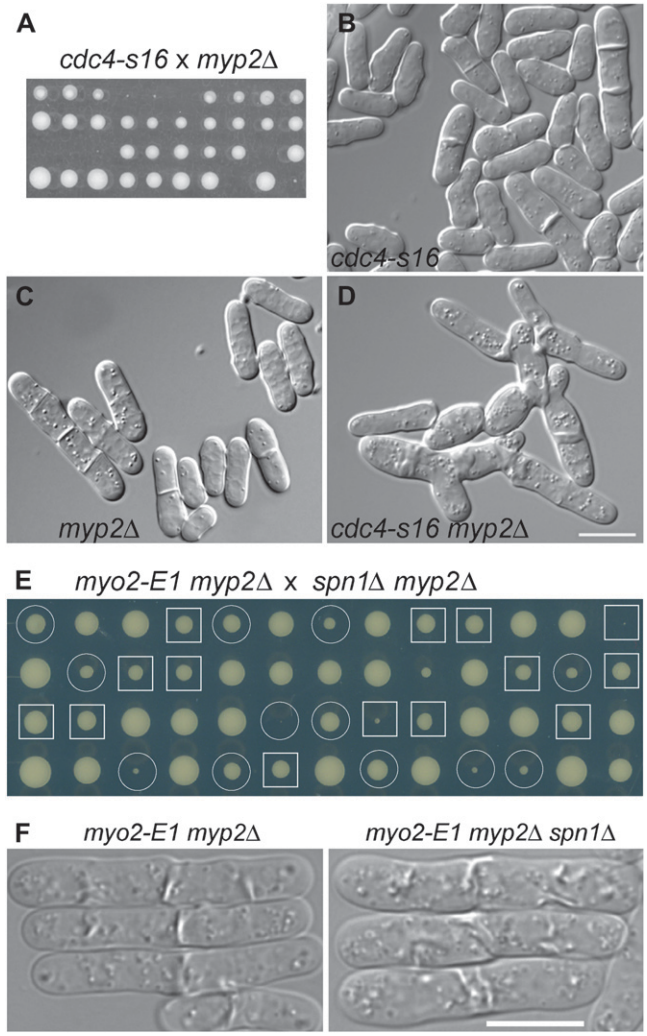

Figure 2.—Genetic interactions among AMR and septin mutations. (A–D) Synthetic lethality between *cdc4-s16* and *myp2*Δ. (A) Strains JW400 (*cdc4-s16*) and TP5 (*myp2*Δ) were crossed, and tetrads were dissected and incubated on YE5S medium at 32° for 6 days. Nine tetrads were tetratypes (*i.e.*, contained one wild-type spore, two single-mutant spores, and one inviable or very slow-growing double-mutant spore) and one was a parental ditype (*i.e.*, each spore was a single mutant and thus grew well). (B–D) DIC micrographs of aberrant cytokinesis in strains JW400, TP5, and JW345 (*cdc4-s16 myp2*Δ). Cells were grown overnight in YE5S liquid medium at 30°. Bar (B–D), 10 μm. (E and F) Absence of synthetic interaction between *myo2-E1 myp2*Δ and *spn1*Δ. (E) Strains TP90 (*myo2-E1 myp2*Δ) and JW380 (*myp2*Δ *spn1*Δ) were crossed, and tetrads were dissected and incubated on a YE5S plate at 25° for 7 days. Circles, *myo2-E1 myp2*Δ segregants; squares, *myo2-E1 myp2*Δ *spn1*Δ segregants. (F) Cells of strains TP90 and JW2560 were grown exponentially in YE5S liquid medium at 25°, shifted to 36° for 4 hr, and examined. Bar, 10 μm.

characterized further. Under *nmt1*-repressing conditions, the *s34* and *s44* single mutants and an *81nmt1-spn1*$^+$ *81nmt1-spn4*$^+$ strain all grew reasonably well, but the triple mutants grew very poorly (Figure 1, F and G), and 24 hr after a shift from inducing to repressing conditions, 5% of 670 *s34* cells, 11% of 1218 *s44* cells, 71% of 820 *s34 81nmt1-spn1*$^+$ *81nmt1-spn4*$^+$ cells, and

76% of 779 *s44 81nmt1-spn1*$^+$ *81nmt1-spn4*$^+$ cells appeared shrunken or lysed (Figure 1, J, K, N, and O). These phenotypes appeared similar at temperatures ranging from 18 to 36°. We also examined the phenotypes of *s34 spn*Δ and *s44 spn*Δ strains produced by crosses among strains JW403, JW289, JW295, and JW406. In each case, most double-mutant segregants arrested as a single elongated cell, while some formed microcolonies with many lysed cells. Inclusion of 1.2 M sorbitol in the medium partially rescued the phenotype and allowed the recovery of viable *s34 spn*Δ and *s44 spn*Δ strains.

To examine the cell-lysis phenotype more closely, we performed time-lapse microscopy on an *s34 spn1*Δ strain (Figure 3A; supporting information, File S1). When *s34 81nmt1-spn1*$^+$ *81nmt1-spn4*$^+$ (JW402) and *s44 81nmt1-spn1*$^+$ *81nmt1-spn4*$^+$ (W405) strains were grown under repressing conditions, they gave very similar results. In the starting populations, some cells had already lysed (*e.g.*, Figure 3A, time 0, lower right) and others were present as short chains of unseparated daughter cells (*e.g.*, Figure 3A, time 0, cells a, b, and c), reflecting the delay in cell separation due to the lack of septins. The time-lapse observations suggested that cell lysis occurred primarily or exclusively during attempted cell separation and in several different patterns. In some cases, only one of the two separating cells lysed [Figure 3A, cell a (time 00:52:00) and cell b (time 00:14:00)]; in other cases, both daughter cells lysed simultaneously (Figure 3A, cell b, compare times 00:52:00 and 00:52:30) or sequentially within ~30 sec (Figure 3A, cell c, time 01:09:00 to time 01:10:30). Interestingly, in some cases, a cell lying between two lysed cells remained intact throughout the entire period of observation (Figure 3A, cell b, second compartment from the left). Although most cells lysed when they attempted to separate, some cells separated successfully, but slowly (>110 min after the appearance of the septum, in contrast to ~50–60 min for the septation-to-separation interval in wild-type cells) (Figure 3A, cell a, right-hand cells, 01:48:00). During the following 5 hr, the daughter cells that had not lysed continued to grow and formed new septa without lysis, supporting the hypothesis that lysis occurred only at the time of attempted cell separation.

Although a few percent of cells harboring only septin mutations also lyse (Figure 1, C and D; Table 3), the strong synthetic phenotypes observed with *s34* and *s44* suggested that the phenotypes due to these mutations did not result from an interference with septin localization or function. Consistent with this interpretation, it appeared that the septins localized normally to the division site in *s34* and *s44* mutant cells (Figure 3B).

To identify the genes containing *s34* and *s44*, we first screened genomic DNA libraries for plasmids that could rescue the phenotypes of *s34 spn1 spn4* and *s44 spn1 spn4* strains. With *s34*, we recovered 13 plasmids whose inserts were mapped to six distinct chromosomal locations by restriction digestion and partial sequencing (Table 4). With *s44*, we recovered 23 plasmids whose inserts mapped to three distinct chromosomal locations that were a subset of those isolated with *s34* (Table 4). Remarkably, the genes contained in these plasmids all appear to represent components of an *S. pombe*

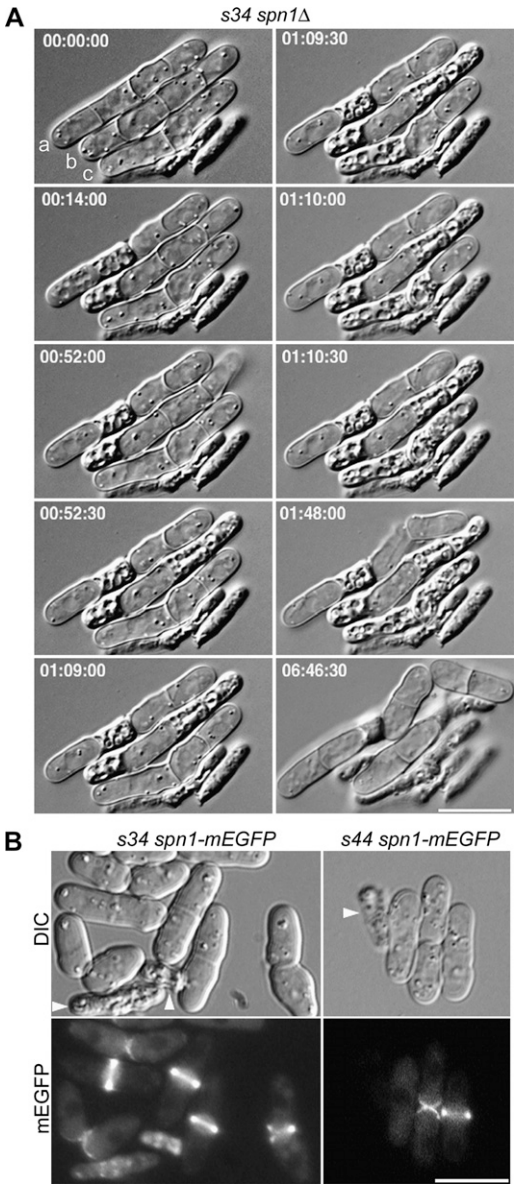

Figure 3.—Cell-lysis phenotype of the *s34 spn1* double mutant and normal septin localization in the *s34* and *s44* mutants. (A) Lysis during cell separation in *s34 spn1*Δ strain JW374. Cells were grown to exponential phase in EMM5S + 1 M sorbitol at 30°, washed twice in EMM5S, and observed by time-lapse DIC photomicroscopy on EMM5S + 25% gelatin at 24° (see materials and methods). Selected images are shown from a series recorded at 30-sec intervals; times are indicated in hours, minutes, and seconds. Cells are labeled for reference in the text. The entire series can be viewed in File S1. (B) Localization of Spn1 in *s34 spn1-mEGFP* strain JW1126 (left panels) and *s44 spn1-mEGFP* strain JW1125 (right panels). Cells were grown in YE5S at 25° and observed by DIC and fluorescence microscopy. Arrowheads in B, lysed cells. Bars, 10 μm.

TABLE 3

Accumulation of cells with abnormal numbers and/or structures of septa in various mutant strains

Strain	No. of cells scored	% of cells with the indicated number of septa[a]					% of lysed or shrunken cells[b]	% of cells with thick walls or septa[c]
		0	1	2	3	≥4		
Wild type	434	86	14	0	0	0	0	0
spn4Δ	487	29	56	5	3	0	7	0
3nmt1-rgf1	434	45	50	4	1	0	0	16[d]
3nmt1-rgf2	400	39	44	9	6	3	0	73[d]
3nmt1-rgf3	363	33	53	9	4	1	0	5[d]
ng124	688	58	31	8	2	1	1	0
ng124 spn1Δ	564	3	32	16	23	16	17[d] + 10	0
mid2Δ	400	37	58	1	2	0	2	0
mid2Δ spn4Δ	465	52	44	1	1	0	2	0
ng124 mid2Δ	613	3	52	13	29	3	17[d]	0
spn1Δ	1240	45	50	2	3	0	ND	ND
scw1Δ	1570	54	40	3	2	0.1	ND	ND
scw1Δ spn1Δ	1237	7	47	14	27	5	ND	ND

Cultures were grown to exponential phase in EMM5S liquid medium at either 30° [strains JW81 (wild type), JW295 (*spn4Δ*), JW331 (*ng124*), JW321-1 (*ng124 spn1Δ*), JW430 (*mid2Δ*), JW314 (*mid2Δ spn4Δ*), and JW315 (*ng124 mid2Δ*)] or 25° [strains JW1078 (*3nmt1-rgf1*), JW903 (*3nmt1-rgf2*), JW1081 (*3nmt1-rgf3*), JW290 (*spn1Δ*), DM1274 (*scw1Δ*), and JW2286 (*spn1Δ scw1Δ*)] before staining with Calcofluor and examining by fluorescence microscopy. ND, not determined.

[a] Some counts total <100% because the numbers of septa were not scored in the lysed or shrunken cells.

[b] Whole cells or individual compartments of septated cells appeared to be lysed.

[c] Cells with very bright Calcofluor staining compared to wild-type cells.

[d] In these cases, the numbers of septa in the cells were also scored.

pathway for the maintenance of cell-wall integrity (see DISCUSSION).

Linkage analysis allowed identification of the genes actually containing *s34* and *s44*. *s34* showed linkage (~20 cM) to the *mat* locus, suggesting that it might be an allele of the nearby *bgs1*, a plausible target (see Introduction) of the cell-wall-integrity pathway. Indeed, a cross of strains JW404 and MBY580 revealed tight linkage (~1 cM) between the *s34* and *bgs1-191* mutations. However, sequencing of genomic DNA from an *s34* strain revealed no mutations in the *bgs1* ORF or its flanking regions. Additional sequencing revealed a single C-to-T substitution at nucleotide 133 of the 1449-bp ORF SPBC19G7.08c, producing a TGA (stop) codon and presumably a null allele. *bgs1-191* has a mutation in codon 277 (Liu *et al.* 1999), 10.18 kb from the putative *s34* mutation, which is consistent with the tight linkage observed. SPBC19G7.08c, now designated *art1*, encodes a previously unstudied arrestin-like protein. The phenotype of an *art1Δ* mutant closely resembles that of the *s34* mutant, and Art1 localizes to the division site (R. Davidson, D. Laporte and J.-Q. Wu, unpublished results), which is consistent with its apparent role in cytokinesis. Further studies of the function of this protein are in progress.

In a cross of *s44* (JW407) and *myo2-E1* (YDM74) strains, no recombinants were observed between these markers in 49 tetrads. Because *myo2* is adjacent to the tandem *rgf3* and *rgf1* (see Table 4), on the *rgf3* side, these data suggested that *s44* is an allele of *rgf3*. However, plasmids containing either *rgf3* or *rgf1* alone can rescue the *s44* mutation (Table 4, footnotes *d* and *i*), and a cross between *s28* and *rgf1Δ* strains also yielded no wild-type recombinants in 20 tetrads (Table 2, line 4). Sequencing genomic DNA from an *s44* strain revealed a single C-to-T substitution in codon 517 of *rgf3*, resulting in a G-to-E substitution in the Rho-GEF domain. Thus, *s44* is an allele of *rgf3*, and *s28* could be in either *rgf3* or *rgf1*.

Independence of septin and Rho-GEF localization to the division site: As described above, mutation *s44* lies in *rgf3*, and *rgf1*$^+$, *rgf2*$^+$, and *rgf3*$^+$, along with *rho1*$^+$, were all recovered as dosage suppressors of *s44* and/or of the phenotypically similar mutation *s34*. The *rgf* genes encode members of the Rho-GEF family (Figure 4A) and have been studied extensively in the past few years (Iwaki *et al.* 2003; Tajadura *et al.* 2004; Morrell-Falvey *et al.* 2005; Mutoh *et al.* 2005; García *et al.* 2006, 2009a,b); all three proteins appear to have Rho1 as a primary or exclusive target. In agreement with these other studies, we found that all three proteins localized to the division site at around the time of cell division (Figure 4, B–D; note that Rgf2, which required overexpression to be visualized, was not studied in as much detail). In addition, Rgf1, but not Rgf2 or Rgf3, localized to a cap at one or both cell poles during interphase (Figure 4D).

Because the septins also assemble at the division site at about the same time as the Rgf proteins, it seemed possible that localization of the Rgf proteins to the division site would depend on a septin scaffold (see Introduction). However, Rgf1 and Rgf3 appeared to localize normally to the division site at the appropriate

TABLE 4

Rescue of mutations *s34* and *s44* by plasmid-borne genes

Mutant	Chromosome region[a]	No. of plasmids	Presumed relevant gene(s)
s34	IIL (C30B4 and C3D6)	1	*wsc1* (homolog of *S. cerevisiae WSC* genes, which encode sensors of surface stress)[b]
	IR (C1006)	1	*rgf2* (encodes a Rho-GEF)[c]
	IIICEN (C645)	2	*rgf1* and *rgf3* (encode Rho-GEFs)[d]
	IR (C1F7)	4	*rho1*[e]
	IIR (C1289)	3	*pob1* (encodes homolog of *S. cerevisiae* Boi1 and Boi2, involved in Rho protein signal transduction)[f]
	IIR (C12D12)	2	*pck2* (encodes a protein kinase C)[g]
s44	IIL (C30B4 and C3D6)	1	*wsc1* (see above)[h]
	IIICEN (C645)	3	*rgf1* and *rgf3* (see above)[i]
	IR (C1F7)	19	*rho1*[j]

Plasmids were recovered during attempts to clone the genes containing mutations *s34* and *s44* (see MATERIALS AND METHODS).

[a] The chromosome arm and relevant cosmid(s) from the *S. pombe* genome project are indicated.

[b] The plasmid contains the entire 1125-bp (no introns) ORF SPBC30B4.01c [designated *wsc1* on the basis of the similarity of its product to the *S. cerevisiae* Wsc proteins (LEVIN 2005)] plus ~2.1 kb of downstream flanking sequence; the 1326 bp between *wsc1* and the uncharacterized ORF SPBC30B4.02c; and the C-terminal 822 bp (~39%) of SPBC30B4.02c, which seems unlikely to be relevant. The 2.1 kb downstream of *wsc1* contains no known or predicted genes.

[c] The plasmid contains the entire 3576-bp coding region (including two introns totaling 99 bp) of *rgf2* (SPAC1006.06); the 729 bp between *rgf2* and the divergently transcribed *och1* (SPAC1006.5c); the entire 1191-bp (no introns) ORF of *och1* (encoding an α-1,6-mannosyltransferase) plus 314 bp of downstream flanking sequence; the 883 bp between *rgf2* and ORF SPAC1006.07; and the N-terminal 337 bp (~29%) of this ORF (which encodes a putative translation factor). *och1* and SPAC1006.07 seem unlikely to be relevant.

[d] One plasmid contains the entire 3828-bp (no introns) *rgf3* (SPCC645.06c) plus ~850 bp of upstream sequence; the 1355 bp between *rgf3* and *myo2* (SPCC645.05c); the N-terminal 2307 bp of the 4581-bp (no introns) *myo2* ORF; but no part of *rgf1* (SPCC645.07). The other plasmid contains the N-terminal 1229 bp (~32%) of *rgf3* (which does not include the Rho-GEF domain); the 1051 bp between *rgf3* and the divergently transcribed *rgf1*; and the N-terminal 3959 bp of the 4051-bp coding region (including one 46-bp intron) of *rgf1*.

[e] Each plasmid has one or the other of two distinct inserts, each of which contains the entire 940-bp coding region (including one 331-bp intron) of *rho1* (SPAC1F7.04) plus flanking sequences. The region of overlap between the inserts contains no other known or predicted genes.

[f] The plasmids have three distinct inserts, each of which contains the entire 2811-bp coding region (including one 195-bp intron) of *pob1* (SPBC1289.04c) plus flanking sequences. The region of overlap between the inserts contains no other known or predicted genes.

[g] The region shared by the two plasmid inserts contains the entire 3051-bp (no introns) ORF of *pck2* (SPBC12D12.04c); the entire 933-bp coding region (including five introns totaling 291 bp) of *rev7* (SPBC12D12.09, encoding a putative DNA polymerase ζ); and the C-terminal 43 bp (~3%) and 306 bp (~24%) of *cct1* (SPBC12D12.03) and SPBC12D12.05c, respectively, which seem unlikely to be relevant. [Note that the SPBC12D12.09 ORF number is out of sequence because this gene was missed during initial annotation (V. WOOD, personal communication).]

[h] The plasmid contains the C-terminal 797 bp (~71%) of the *wsc1* ORF (see footnote *b*) plus 1584 bp of downstream sequence that contains no other known or predicted gene.

[i] The three plasmids have identical inserts that contain only an internal 1930-bp fragment (lacking the N-terminal 1681 bp and the C-terminal 440 bp) of the *rgf1* coding sequence (see footnote *d*). This fragment contains the Rho-GEF domain. In addition, the two *rgf1/rgf3* plasmids isolated in attempting to clone the *s34* gene (see footnote *d*) were also able to rescue the phenotype of the *s44* strain JW405.

[j] The plasmids have at least six distinct inserts, all of which contain the entire *rho1* gene (see footnote *e*) plus flanking sequences. The region of overlap between the inserts contains no other known or predicted genes.

times in a septin mutant (Figure 4, D and E; File S2). In addition, the Rgf1 and Rgf3 rings constricted as the furrow ingressed during cytokinesis in wild-type cells, whereas the septin rings did not (Figure 4B).

In further agreement with the published studies, we found that overexpression of the Rgf proteins produced cells with multiple and/or aberrant septa (Figure 4F; Table 3). Because this phenotype resembled that of septin mutants, it seemed possible that overexpression of the Rgf proteins interfered with septin localization. However, Spn1 appeared to localize normally when either Rgf1 or Rgf3 was overexpressed (Figure 4F). In addition, the mild vegetative phenotypes of *rgf1*Δ and *rgf2*Δ mutants (MORRELL-FALVEY *et al.* 2005; MUTOH *et al.* 2005; GARCÍA *et al.* 2006, 2009b; our unpublished data) suggest that septin function is not perturbed by a lack of Rgf1 or Rgf2, and septins also appeared to localize normally in an *rgf3-s44* mutant strain (Figure 3B).

Taken together, the data suggest that the pathway involving Rho1 and its GEFs functions independently of that involving the septins, although the two pathways may complement each other in function.

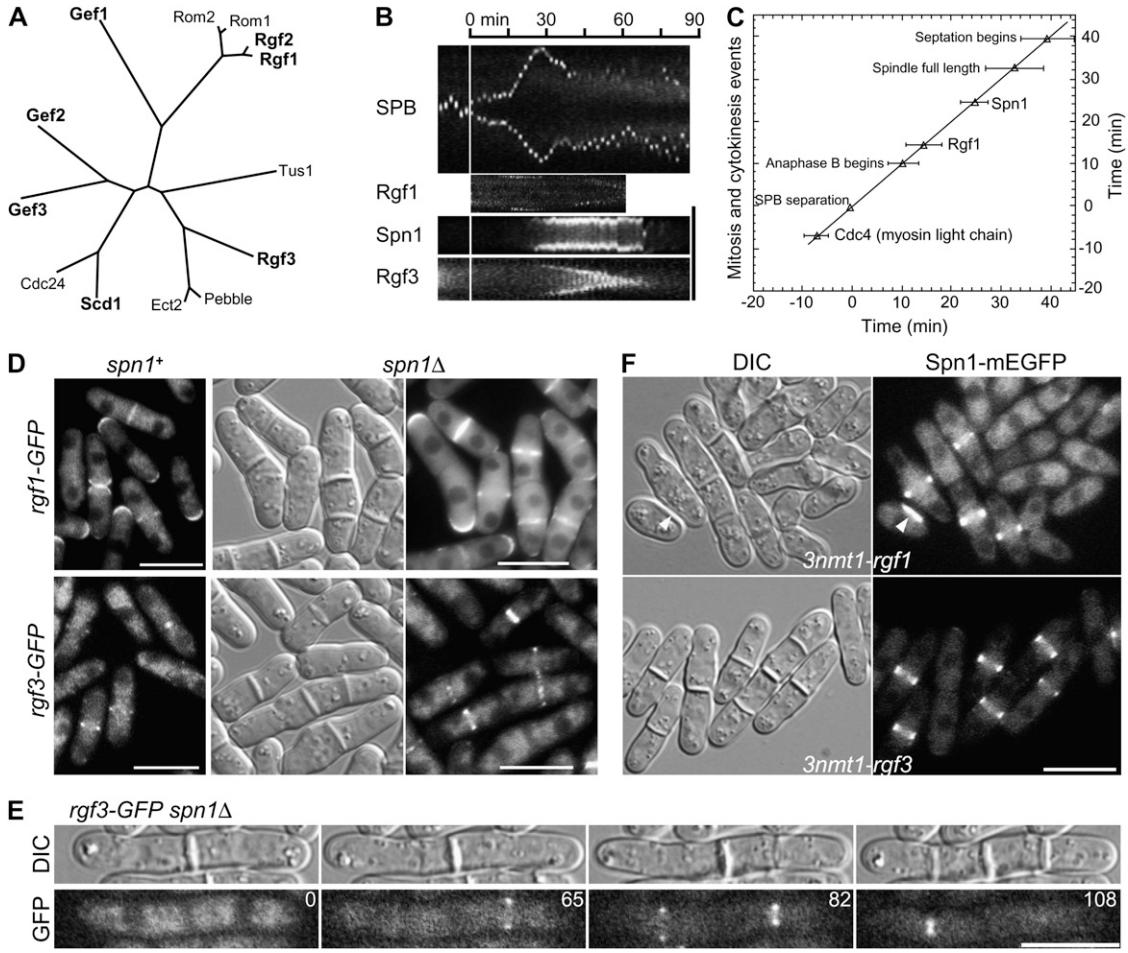

Figure 4.—Independence of septin and Rho-GEF localization to the division site. (A) Relationships among Rho-GEFs from *S. pombe* (http://www.genedb.org/genedb/pombe/) and *S. cerevisiae* (http://www.yeastgenome.org/), as well as a Rho-GEF from Drosophila (Pebble) and one from humans (ECT2) that have also been implicated in cytokinesis (Miki *et al.* 1993; Prokopenko *et al.* 1999; Tatsumoto *et al.* 1999; O'Keefe *et al.* 2001; Giansanti *et al.* 2004; Shandala *et al.* 2004). Clustal W was used to align the Rho-GEF domains and then derive a phylogenetic tree based on this alignment. (B and C) Localization and dynamic behavior of Rgf1 and Rgf3 at the division site in relation to the septins and other cell-division markers in wild-type cells. Strains JW1113 (*spn1-mEGFP sad1-mRFP1*), JW1124 (*rgf1-GFP sad1-mRFP1*), and JW1131 (*rgf3-mEGFP sad1-mRFP1*) were filmed in EMM5S at 25°. (B) Kymographs constructed using Image J and the movements of the spindle-pole bodies (SPBs), as marked by Sad1-mRFP1, to allow alignment at the time of SPB separation (vertical line). For the SPB, a 13.7-μm slit parallel to the long axis of the cell was used; for the other proteins, a 4.1-μm slit across the midplane of the cell was used. (C) Timeline showing the initial appearance of Spn1 ($n = 8$ cells) and Rgf1 ($n = 8$) at the division site. SPB separation is defined as time zero, and the mean time of appearance of each protein (±1 standard deviation) is plotted. The beginning of septation was scored by DIC. The data for Cdc4 are from Wu *et al.* (2003). (D and E) Tests of possible septin dependence of Rho-GEF localization. (D) Strains JW1124 (*rgf1-GFP*), JW1139 (*rgf1-GFP spn1*Δ), JW1131 (*rgf3-mEGFP*), and JW1128 (*rgf3-GFP spn1*Δ) were grown in EMM5S (JW1124 and JW1131) or YE5S (JW1139 and JW1128) medium at 25° and examined by DIC and/or fluorescence microscopy. (E) Strain JW1128 was examined by time-lapse microscopy. Selected DIC and GFP images (elapsed times given in minutes) are shown from a series recorded at 1-min intervals. The entire series can be viewed in File S2. (F) Cell morphology and septin localization in cells overexpressing Rgf1 or Rgf3. Strains JW1123 (*spn1-mEGFP 3nmt1-rgf1*) and JW1122 (*spn1-mEGFP 3nmt1-rgf3*) were grown under inducing conditions (EMM5S medium) for 24 hr at 25°; paired DIC and fluorescence images are shown. Arrowheads indicate a cell with a region of thickened cell wall. Bars (B and D–F), 10 μm.

A mutation that may identify a pathway parallel to that of the septins: The original *ng124 81nmt1-spn1*$^+$ *81nmt1-spn4*$^+$ triple mutant had a subtle growth defect but a conspicuous cell-separation delay. After backcrossing, *ng124* single mutants grew well (Figure 1H) but had a mild cell-separation delay like that of septin mutants (Figure 1L; Figure 5B; Table 3). An *ng124 spn1*Δ double mutant also grew reasonably well (Figure 1H) but displayed a much more severe cell-separation delay, such that nearly all cells contained one or more septa, and some cells had lysed (Figure 1P; Table 3). This phenotype was similar at temperatures ranging from 18° to 36° but was more dramatic on solid media (where many cells had up to seven or nine septa) than in liquid media. In a typical time-lapse series (Figure 5A; File S3), no successful cell separation was observed during >4.5 hr

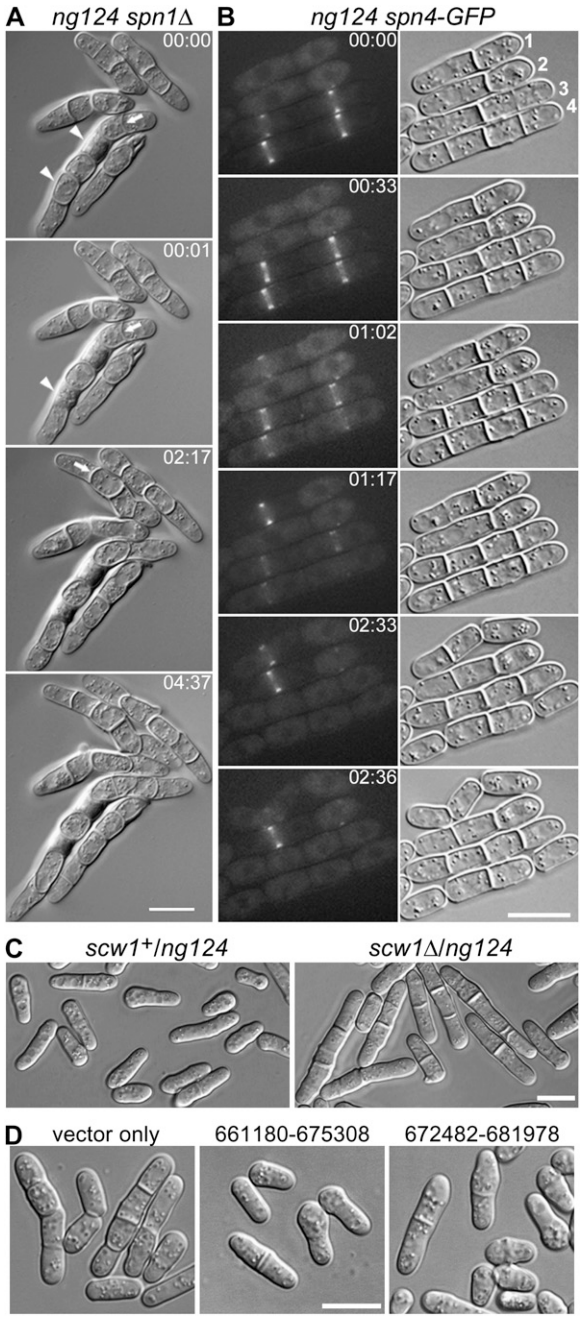

Figure 5.—Characterization of mutant *ng124* and its identification as an allele of *scw1*. (A and B) Selected DIC and fluorescence images are shown from time-lapse-microscopy series recorded at 1- or 2-min intervals; times are indicated in hours and minutes. The entire series can be viewed in File S3 and File S4. (A) Cell-separation defect in an *ng124 spn1*Δ double mutant. Strain JW321-1 was grown in YE5S medium at 30° and observed on YE5S at 24°. Arrows indicate abnormal-shaped septa; arrowheads indicate cells that appear to have lysed. (B) Apparently normal septin localization and variable cell-separation delay in an *ng124* single mutant. Strain JW320 (*ng124 spn4-GFP*) was grown in EMM5S at 23° and observed on EMM5S at 24°. Cells are numbered for reference in the text. (C) Noncomplementation of *ng124* and *scw1*Δ in diploid cells. Diploid strains JW2613 (*scw1*$^+$*/ng124*) and JW2614 (*scw1*Δ*/ng124*) were observed by DIC microscopy after growth on YE5S-Ade at 25°. (D) Rescue of *ng124* by plasmids containing *scw1*$^+$. Strain JW2155 (*ng124*) was transformed with empty vector or with chromosome III plasmids that contain only *scw1* (nucleotides 673,476–675,161) in their region of overlap (nucleotides 672,482–675,308), grown in EMM5S-Leu medium at 25°, and observed by DIC microscopy. Bars, 10 μm.

of filming, although some older septa became very abnormal in shape (Figure 5A, arrows) and some cells lysed (Figure 5A, arrowheads; Table 3).

Although the *ng124* single-mutant phenotype resembled that of septin mutants, the synthetic phenotype suggested that *ng124* did not interfere with septin localization or function. Consistent with this interpretation, time-lapse and confocal microscopy showed that the septins appeared at the presumptive division site at the normal time (just before septum formation) and in the normal double-ring configuration in an *ng124* mutant [Figure 5B, particularly cell 3 at 00:00 (note septin rings visible at two sites where septa are just beginning to form) and cell 1 at 01:02 and 01:17; Figure 6, A–C; File S4, File S5, and File S6]. In addition, although the times elapsing between septum formation and cell separation were highly variable (Figure 5B: compare cell 1 at 01:17, 02:33, and 02:36 to cells 3 and 4 at 00:00 and 02:36), the septins appeared to disassemble from the septation site at approximately the normal time (*i.e.*, when cell separation would normally occur) regardless of whether or not cell separation occurred on schedule at that particular site (Figure 5B: compare cell 1 at 01:17, 02:33, and 02:36 to cells 3 and 4 at 00:00, 01:02, 01:17, and 02:33).

*mid2*Δ mutants also have a cell-separation delay resembling that of septin mutants (Berlin *et al.* 2003; Tasto *et al.* 2003; Figure 6H; Table 3). Like *ng124 spn*Δ double mutants, an *ng124 mid2*Δ double mutant has a strong synthetic phenotype, with more frequent and longer chains of cells than found in the single mutants (Figure 6I; Table 3). In striking contrast, *spn4Δ mid2*Δ double mutants display no obvious synthetic phenotype and resemble the two single mutants (Berlin *et al.* 2003; Tasto *et al.* 2003; Figure 6J; Table 3). These observations suggest that Mid2 and the septins function together in one pathway while the product of the gene identified by *ng124* functions in a distinct and perhaps redundant pathway. Observations of protein localization are consistent with this hypothesis. Mid2 and the septins colocalize interdependently to a double ring at the division site (Berlin *et al.* 2003; Tasto *et al.* 2003; Figure 6, A, B, D, E, and G; File S5, File S7, and File S8). In contrast, both the septins (see above) and Mid2 (Figure 6F; File S9) appear to localize almost normally in *ng124* mutant strains.

After failing to identify the *ng124*-containing gene by plasmid rescue using the available libraries, we used positional cloning (see materials and methods), which suggested that *ng124* might be an allele of *scw1*. We confirmed this identification by showing (i) that

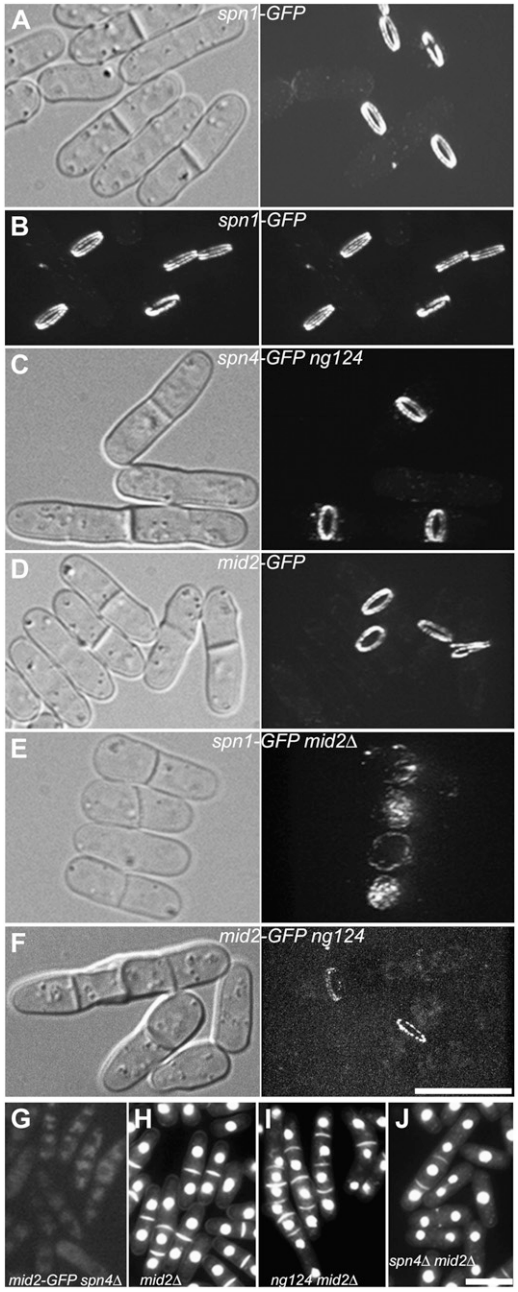

FIGURE 6.—Functional relationships among the septins, Mid2, and Scw1. Cells were observed by spinning-disk confocal microscopy (A–F) or by wide-field fluorescence microscopy (G–J) after growth in YE5S at 25° (A–G) or EMM5S at 30° (H–J). In each case, the cells shown are representative of large numbers of cells examined. For the fluorescence images in A–F, stacks of 70 *z*-sections (0.07-μm) were captured using the same laser power and acquisition settings and projected into three-dimensional images using Image J (see MATERIALS AND METHODS). Selected views of the three-dimensional images are shown with corresponding DIC images; the entire series can be viewed in File S5, File S6, File S7, File S8, and File S9. (A and B) Localization of Spn1-GFP in wild-type strain JW306. The images in B are a stereo pair. (C) Localization of Spn4-GFP in *scw1-ng124* strain JW320. (D) Localization of Mid2-GFP in wild-type strain JW326. (E) Localization of Spn1-GFP in *mid2*Δ strain JW318. (F) Localization of Mid2-GFP in *scw1-ng124* strain JW1203. (G) Localization of Mid2-GFP in *spn4*Δ strain JW332. (H–J) Mutant phenotypes of strains (H) JW430 (*mid2*Δ), (I) JW315 (*scw1-ng124 mid2*Δ), and (J) JW314 (*spn4*Δ *mid2*Δ). Cells were stained with bisbenzimide to view nuclei and septa. Bars, 10 μm.

ng124 and an *scw1* deletion fail to complement in a diploid (Figure 5C), (ii) that two plasmids containing only *scw1*$^+$ in their region of overlap rescue the *ng124* phenotype (Figure 5D), and (iii) that an *ng124* strain has a G-to-A mutation (producing a stop codon) in codon 276 of the 562-codon *scw1* ORF. Like *ng124*, *scw1*Δ has a strong synthetic effect with an *spn*Δ mutation (Table 3). Consistent with previous evidence (KARAGIANNIS *et al.* 2002; JIN and MCCOLLUM 2003), our data suggest that Scw1 does not function in the AMR pathway: AMR formation and constriction had no obvious defects in an *scw1*Δ strain, and synthetic growth defects were seen between *scw1-ng124* and both *myo2-E1* and *cdc4-s16* (data not shown). Taken together, our data suggest that Scw1 functions in parallel to the AMR and the septin/Mid2 pathway to promote cell division (see DISCUSSION).

DISCUSSION

The roles of the septins remain among the central puzzles of cytokinesis. These proteins localize to the division site in every fungal and animal cell type examined, and they are essential for successful cytokinesis in many cases. In other cell types, however, the septins are dispensable for cytokinesis (see Introduction). One possible resolution of this paradox is that a conserved septin role in cytokinesis can also be filled effectively in some cell types by a redundant protein(s) or pathway. In an attempt to identify such a redundant system, we conducted a genetic screen in *S. pombe* for mutations that are lethal in combination with a loss of septins. Although the results obtained have not yet solved the central problem, they have nonetheless shed considerable light on the role of the septins and other aspects of *S. pombe* cell division. In particular, the results suggest strongly that a septin/Mid2-dependent pathway functions in parallel with several other pathways to ensure successful cytokinesis, septation, and cell separation (Figure 7).

Genetic interaction between the septins and Cdc4: One of the synthetic-lethal mutants contained a hypomorphic or neomorphic allele, *cdc4-s16*, of the essential gene *cdc4*. Cdc4 is thought to function as a light chain for both type II myosin heavy chains, Myo2 and Myp2 (NAQVI *et al.* 1999; MOTEGI *et al.* 2000; D'SOUZA *et al.* 2001; LORD and POLLARD 2004). Myo2 and Myp2 appear to be partially redundant for function in cytokinesis but are specialized such that *myo2* is an essential gene whereas *myp2* is essential only under particular growth conditions (BEZANILLA and POLLARD 2000; MOTEGI *et al.* 2000). We found that *cdc4-s16* and *myp2*Δ, like *myo2-E1* and *myp2*Δ, are synthetically lethal (Figure 2, A–D); this

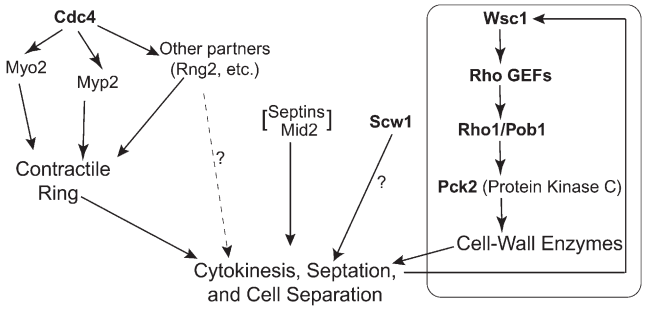

Figure 7.—Possible organization of parallel pathways that cooperate to allow successful cytokinesis, septation, and cell separation in fission yeast, as suggested by the results of this study. Boldface type indicates the products of genes identified in this study. The cell-wall-integrity pathway headed by Wsc1 contains additional proteins beyond those shown here; the degree to which this pathway participates in unperturbed cytokinesis is not known. See text for details.

suggests that *cdc4-s16* compromises the function of Myo2, consistent with the other strong evidence that Cdc4 is a light chain for Myo2. We also found that *cdc4-s16* and *myo2-E1* are synthetically lethal (see RESULTS). Similar observations have been made with two other *cdc4* alleles (Naqvi *et al.* 1999). Because *myp2*Δ is also synthetically lethal with *myo2-E1*, these results suggest that *cdc4-s16* compromises the function of Myp2, consistent with the other evidence that Cdc4 is a light chain for Myp2. The hypothesis that *cdc4-s16* generally compromises myosin II function in the AMR is also supported by the observation that *cdc4-s16*, like a variety of other mutations affecting the AMR (Mishra *et al.* 2004), is synthetically lethal with a *clp1*Δ protein phosphatase mutation (our unpublished results). However, other explanations for the *cdc4-s16 myo2-E1* synthetic lethality remain possible. For example, it might result from an exacerbation by *cdc4-s16* of the *myo2-E1* effect on Myo2 function or from a partial loss of AMR function (due to *myo2-E1*) combined with a partial loss of some other Cdc4-mediated function (see below).

It is possible that the synthetic lethality between *cdc4-s16* and septin mutations arises because the lack of septins further compromises the function of Myo2 or Myp2. However, we could obtain no evidence to support these possibilities (see RESULTS and Figure 2, E and F). Another hypothesis is that *cdc4-s16* also compromises the function of some other Cdc4-binding partner that is part of a pathway that normally complements the pathway involving the septins. In this regard, it should be noted that there is good evidence that both *S. pombe* Cdc4 and its *S. cerevisiae* counterpart, Mlc1, interact with partners other than the type II myosin heavy chains and that these other partners are also important for cytokinesis (Stevens and Davis 1998; Boyne *et al.* 2000; Shannon and Li 2000; Desautels *et al.* 2001; D'Souza *et al.* 2001; Motegi *et al.* 2001; Win *et al.* 2001; Lord and Pollard 2004; Luo *et al.* 2004; Mulvihill *et al.* 2006; Park *et al.* 2009). The other partners include type V myosin heavy chains (*S. pombe* Myo51 and perhaps Myo52; *S. cerevisiae* Myo2), which are thought to be involved in the delivery of new plasma-membrane and cell-wall materials to the division site or (for Myo51) in some unknown function within the AMR; IQGAPs (*S. pombe* Rng2 and *S. cerevisiae* Iqg1), which may also be involved in plasma-membrane and cell-wall formation in addition to their roles in AMR function; and a phosphatidylinositol 4-kinase (*S. pombe* Pik1). From this hypothesis, it would be expected that the *spn*Δ *cdc4-s16* synthetic lethality would be paralleled by strong synthetic effects between *spn*Δ and mutations affecting these other binding partners. However, we found no detectable synthetic effect between *spn1-Δ2* and *myo51*Δ, *rng2-D5* (Balasubramanian *et al.* 1998; Eng *et al.* 1998), *rng2-346* (Chang *et al.* 1996), or *pik1Δ*$^{117\text{-}198}$ (Onishi *et al.* 2010) and only a weak synthetic effect between *spn1-Δ2* and *myo52*Δ (our unpublished results).

Thus, the most attractive hypothesis to explain the available data is that the septins are involved in a pathway whose function normally complements that of the AMR (Figure 7). If *cdc4-s16* indeed compromises the function of both Myo2 and Myp2, its overall effect on the AMR may be sufficiently severe to produce synthetic lethality in combination with a loss of the septin-dependent pathway. However, given the lack of an obvious synthetic effect in a *myo2-E1 myp2Δ spn1*Δ triple mutant (Figure 2, E and F), this hypothesis also requires us to suppose that the *cdc4-s16* effect on the AMR is more complex than a simple loss of myosin II function. Thus, it remains possible that the synthetic lethality between *cdc4-s16* and septin mutations reflects an effect of *cdc4-s16* on some unknown Cdc4 binding partner; further studies will be necessary to clarify this situation. In any case, however, the results do demonstrate the important point that the septins have a role in *S. pombe* cytokinesis that becomes critical under some conditions.

Role of a cell-wall-integrity pathway in cytokinesis and in the viability of septin mutants: Five of the remaining synthetic-lethal mutants had very similar cell-lysis phenotypes and mapped to two loci. Our attempts to clone the corresponding genes led to the isolation of numerous dosage suppressors (Table 4), in addition to the gene (*rgf3*) that actually contains the *s44* mutation. Given what is known about the functions of Rho1, the Rho-GEFs, Pck2, and Pob1 in *S. pombe* (see Introduction; Toya *et al.* 1999; Rincón *et al.* 2009) and about the well-studied cell-wall-integrity pathway in *S. cerevisiae* (Levin 2005), it seems clear that the seven genes identified as dosage suppressors all represent components of a similar pathway in *S. pombe*, as also described, in part, in several other recent studies (Madrid *et al.* 2006, 2007; Barba *et al.* 2008; and references cited therein). In this model, Wsc1 is a sensor of cell-wall damage that works through one or more of the Rho-GEFs Rgf1, Rgf2, and Rgf3 to activate Rho1.

(Note that a Rho-GEF that does not normally function in this pathway might be able to do so when overexpressed.) Activated Rho1 (with its function facilitated by Pob1) would trigger several responses that would help to correct the damage, including the activation of Pck2, which would in turn activate a MAP-kinase pathway (Madrid *et al.* 2006; Barba *et al.* 2008) that would lead to increased expression of genes whose products are involved in the repair of cell-wall damage.

The identification of *s34* as a mutation in SPBC19G7.08c/*art1*, which encodes one of a family of at least eight arrestin-related proteins in *S. pombe*, was unexpected and interesting. The arrestins were originally identified for their roles in the regulation of G-protein-coupled receptors but were later found to function more broadly in the endocytic regulation of plasma-membrane proteins (Lefkowitz *et al.* 2006; Moore *et al.* 2007). To our knowledge, the *S. pombe* arrestin-related proteins have not been studied previously, except for one involved in regulating meiotic entry (Matsuyama *et al.* 2000), but their counterparts in *S. cerevisiae* have been reported to function as ubiquitin-ligase adaptors that regulate endocytosis of cell-surface proteins (Lin *et al.* 2008; Nikko and Pelham 2009). The observations that Art1 localizes to the cell-division site, that the *art1-s34* mutant phenotype is essentially identical to that of *rgf3-s44*, and that *art1-s34* is rescued by overexpression of seven different genes encoding components of the cell-wall-integrity pathway all suggest strongly that Art1 also functions as a component of this pathway. Thus, our continuing studies of Art1 function should help to elucidate not only the mechanisms for ensuring cell-wall integrity during cell division but also the functions of this family of proteins.

In any case, a plausible interpretation of our observations to date is as follows (Figure 7). In the absence of the septins, the septum that forms has defects, the localization of lytic enzymes that function in cell separation is perturbed (see Martín-Cuadrado *et al.* 2005; Sipiczki 2007), or both. If the cell-wall-integrity pathway is functioning normally, the problems can be recognized and repaired, and the cells usually divide successfully (albeit typically after a significant delay). The *rgf3-s44* and *art1-s34* mutations compromise the cell-wall-integrity pathway, so that the damage resulting from the absence of septins is not repaired efficiently, and thus the cells often lyse when they attempt to separate. However, overexpression of components of the cell-wall-integrity pathway can activate it sufficiently that the absence of other components is overcome, cell-wall problems are repaired, and cell division usually occurs successfully. Given that the MAP-kinase pathway appears to be partially activated in every cell cycle (Madrid *et al.* 2007), the absence of the septins may only exacerbate a problem that exists even in normal cells as they attempt the delicate task of forming a septum and then degrading it enough to separate, without leaving a weakness that would lead to lysis driven by the intracellular turgor pressure.

In this context, it is also of interest that, in the *scw1-ng124* mutant, some cells underwent seemingly normal cell separation long after the septins had disassembled from the septal region (Figure 5B, cell 1 at 00:00, 01:17, and 2:33). This observation suggests that the septins may not play a direct role in cell separation and that some checkpoint mechanism delays attempted cell separation in an *scw1-ng124* (or *spn*Δ) mutant until septal-wall defects have been repaired.

The possibility of a pathway redundant with that involving the septins: We began the synthetic-lethal screen with the hope of identifying a functionally redundant pathway that would explain why the septins are not essential for cytokinesis in *S. pombe* and in some other cell types. It is possible that the final mutation identified in our screen, *scw1-ng124*, affects such a pathway (Figure 7). The *scw1-ng124* and *scw1*Δ single mutants have a division delay like that seen in septin or *mid2* mutants, but several observations suggest that Scw1 does not function in the septin/Mid2 pathway. First, although the septins and Mid2 depend on each other for normal localization, both the septins and Mid2 localize essentially normally in an *scw1* mutant (Figure 6). Moreover, although septin and *mid2* mutations have no synthetic effect with each other, *scw1* mutations have strong synthetic effects with each (Figures 5A and 6; Table 3).

Scw1 and *scw1* mutants have been described previously (Karagiannis *et al.* 2002; Jin and McCollum 2003). Scw1 contains a putative RNA-binding domain, and an *S. cerevisiae* homolog, Whi3, indeed binds to a large set of mRNAs (Colomina *et al.* 2008). Because *scw1* mutations can suppress mutations both in the SIN pathway (see Introduction) and in the β-glucan synthase Bgs1, the earlier reports suggested that Scw1 might be a negative regulator of septum formation, so that *scw1* mutations would promote septum formation. However, this interpretation is difficult to reconcile with the observations that the septa in an *scw1* mutant seem to appear with normal timing but then be delayed in separation (Figure 5B) and that this cell-separation delay is enhanced in *scw1 spn* and *scw1 mid2* double mutants (Figures 1P, 5A, and 6I; Table 3). Thus, we suggest that a more likely interpretation of the available information is as follows. Scw1 may promote some step in the initiation of cell separation or a step in septum completion (perhaps not easy to visualize microscopically) that is necessary to initiate cell separation. Thus, the absence of functional Scw1 in an *scw1* mutant would (1) result in multiseptated cell chains, as observed, and (2) rescue SIN mutants and *bgs1-191* by producing a delay in attempted cell separation that would provide more time for SIN- and Bgs1-dependent processes to be completed, so that the daughter cells would separate successfully without lysis. If Scw1 and the septins

function in parallel pathways for completion of the same events, then the strong synthetic effect between *scw1*Δ and *spn*Δ (or *mid2*Δ) mutations could be explained. The identification of proteins and/or mRNAs bound by Scw1 should enable us to explore these possibilities further.

Limitations of the synthetic-lethal screening to date: Because we obtained only one *cdc4* and one *scw1* mutation, our screen was probably not saturated, so that further screening of the same type might identify additional genes of interest. However, it is also possible that the design of the screen limited the genes that we were able to identify. Use of the *nmt1* promoter for synthetic-lethal screening is convenient in the simple comparison of cells grown in the absence and presence of thiamine (Yoon *et al.* 1997; Berry *et al.* 1999; this study). However, the *nmt1* promoters are leaky, and cells retain some level of expression under repressing conditions (Forsburg 1993; Berry *et al.* 1999). Thus, we might have missed some mutations that would have had stronger interactions with septin null mutations. Further synthetic-lethal screening using a different system might be fruitful in providing additional insights into the roles of the septins in cytokinesis in *S. pombe* and thus, presumably, also in other types of cells.

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Cooperation Between the Septins and the Actomyosin Ring and Role of a Cell-Integrity Pathway During Cell Division in Fission Yeast

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FILES S1-S9

Supporting Movies

Files S1-S9 are available for download as .mov files at http://www.genetics.org/cgi/content/full/genetics.110.119842/DC1.

File S1: Cell-lysis phenotype during cell separation in *s34 spn1Δ* strain.

File S2: Septin independence of Rho-GEF Rgf3 localization to the division site. DIC and GFP images of *rgf3-GFP spn1Δ* cells are shown.

File S3: Cell-separation defect in an *ng124 spn1Δ* double mutant.

File S4: Normal septin localization and variable cell-separation delay in an *ng124 spn4-GFP* mutant.

File S5: Localization of Spn1-GFP in wild-type strain.

File S6: Localization of Spn4-GFP in *scw1-ng124* strain.

File S7: Localization of Mid2-GFP in wild-type strain.

File S8: Localization of Spn1-GFP in *mid2* deletion strain.

File S9: Localization of Mid2-GFP in *scw1-ng124* strain.