

# Novel *sfi1* Alleles Uncover Additional Functions for Sfi1p in Bipolar Spindle Assembly and Function

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A variety of spindle and kinetochore defects have been shown to induce a mitotic delay through activation of the spindle checkpoint. With the aim of identifying novel mitotic defects we carried out a *mad1* synthetic lethal screen in budding yeast. In this screen, four novel alleles of *sfi1* were isolated. *SFI1* is an essential gene, previously identified through its interaction with centrin/*CDC31* and shown to be required for spindle pole body (SPB) duplication. The new mutations were all found in the C-terminal domain of Sfi1p, which has no known function, but it is well conserved among budding yeasts. Analysis of the novel *sfi1* mutants, through a combination of light and electron microscopy, revealed duplicated SPBs <0.3  $\mu\text{m}$  apart. Importantly, these SPBs have completed duplication, but they are not separated, suggesting a possible defect in splitting of the bridge. We discuss possible roles for Sfi1p in this step in bipolar spindle assembly.

## INTRODUCTION

The spindle checkpoint acts as a surveillance system and monitors kinetochore–microtubule interactions during mitosis (Musachio and Hardwick, 2002; Cleveland *et al.*, 2003; Taylor *et al.*, 2004). It helps ensure that all sister chromatids are bioriented, with sisters attached to opposite spindle poles, before anaphase onset. This checkpoint is not essential in yeasts; indeed, deletion of the mitotic arrest defective (*MAD*) genes leads to a rather subtle phenotype in the absence of additional perturbation of the mitotic machinery (Warren *et al.*, 2002). However, mouse knockouts of *Mad2* and *Bub3* are early embryonic lethal (Dobles *et al.*, 2000; Kalitsis *et al.*, 2000), and it has been argued that this is because the spindle checkpoint plays a critical role in the timing of mitosis in vertebrates (Meraldi *et al.*, 2004). Efficient RNA interference (RNAi) of *Mad* and *Bub* components in tissue culture shortens mitosis so much that it is catastrophic, because there simply is not enough time to build a spindle and attach all kinetochores properly before anaphase onset (Meraldi *et al.*, 2004; Michel *et al.*, 2004).

The nonessential nature of the yeast *MAD* genes makes them extremely tractable genetically, and allowed us to perform a synthetic lethal screen with the aim of identifying novel mitotic defects that activate the spindle checkpoint.

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Abbreviations used: EM, electron microscopy; MAD, mitotic arrest defective; NAT, nourseothricin; SPB, spindle pole body.

Neither the mitotic mutation nor a *mad1* $\Delta$  on their own kill cells, but when combined they become lethal. Synthetic lethal screens with *mad* mutants have been carried out using the viable haploid gene deletion set, and many interactions found, including a range of nonessential spindle and kinetochore functions (Lee and Spencer, 2004; Daniel *et al.*, 2006). Our screen is unbiased: any essential or nonessential component of the mitotic machinery that can be mutated to cause a defect that is recognized by the spindle checkpoint can be identified. A pilot screen that identified 25 mitotic mutants was conducted (Hardwick, unpublished data), but among them were four novel alleles of *sfi1*.

*SFI1* was initially identified as a suppressor of the *fil1* mutation (Ma *et al.*, 1999), and it has since been demonstrated to be an essential and widely conserved component of centrosomes, which are known in yeast as spindle pole bodies (SPBs) (Kilmartin, 2003).

The budding yeast spindle pole body is a layered structure embedded in the nuclear envelope and is the sole microtubule-organizing center, nucleating both nuclear and cytoplasmic microtubules from its inner and outer plaques, respectively. Associated with one side of the SPB is a structure called the half-bridge, a specialization of nuclear membrane with a layer of material on the cytoplasmic face (O'Toole *et al.*, 1999). The half-bridge serves several important roles during spindle pole body duplication. On initiation of SPB duplication, the half-bridge extends and a small satellite body is formed on the distal cytoplasmic tip (Byers, 1981). The satellite expands to form a duplication plaque, which is then inserted in the membrane by an unknown process, possibly involving contraction of the half-bridge (Adams and Kilmartin, 1999). After the inner plaque assembles on the nuclear face of duplication plaque, the result is two side-by-side SPBs joined by the bridge. The bridge then cleaves or dissociates, allowing the two SPBs to separate and organize opposite ends of the spindle.

One important component of the half-bridge is Cdc31p, the single budding yeast member of the conserved centrin

**Table 1.** Yeast strains used, all of which are derivatives of W303

Yeast strains	
slm65 MATa	<i>sfi1-65, mad1Δ::HIS3, ura3-1, leu2,3-112, his3-11, trp1-1, ade2-1, p(MAD1, URA3, ADE3)</i>
slm120 MATa	<i>sfi1-120, mad1Δ::HIS3, ura3-1, leu2,3-112, his3-11, trp1-1, ade2-1, p(MAD1, URA3, ADE3)</i>
slm229 MATa	<i>sfi1-229, mad1Δ::HIS3, ura3-1, leu2,3-112, his3-11, trp1-1, ade2-1, p(MAD1, URA3, ADE3)</i>
slm273 MATa	<i>sfi1-273, mad1Δ::HIS3, ura3-1, leu2,3-112, his3-11, trp1-1, ade2-1, p(MAD1, URA3, ADE3)</i>
IAY18 MATa	<i>TRP1::SPC42-GFP, spc42Δ1::LEU2, ade2-1, trp1-1, can1-100, leu2,-3, his3-11, ura3</i>
VAY3 MATa	<i>SFI1-YFP::G418, SPC42-CFP::TRP1</i>
VAY20 MATa	<i>sfi1-65, TRP1::SPC42-GFP, ura3-1, leu2,3-112, his3-11, trp1-1, ade2-1, can1-100</i>
VAY21 MATa	<i>sfi1-120, TRP1::SPC42-GFP, ura3-1, leu2,3-112, his3-11, trp1-1, ade2-1, can1-100</i>
VAY22 MATa	<i>sfi1-229, TRP1::SPC42-GFP, ura3-1, leu2,3-112, his3-11, trp1-1, ade2-1, can1-100</i>
VAY23 MATa	<i>sfi1-273, TRP1::SPC42-GFP, ura3-1, leu2,3-112, his3-11, trp1-1, ade2-1, can1-100</i>
VAY24 MATa	<i>TRP1::SPC42-GFP, ura3-1, leu2,3-112, his3-11, trp1-1, ade2-1, can1-100</i>
KH321 MATa	<i>ura3-1, leu2,3-112, his3-11, trp1-1, ade2-1, can1-100, CFIII URA3 SUP11</i>
VAY25 MATa	<i>sfi1-65, ura3-1, leu2,3-112, his3-11, trp1-1, ade2-1, can1-100, CFIII URA3 SUP11</i>
VAY26 MATa	<i>sfi1-120, ura3-1, leu2,3-112, his3-11, trp1-1, ade2-1, can1-100, CFIII URA3 SUP11</i>
VAY27 MATa	<i>sfi1-229, ura3-1, leu2,3-112, his3-11, trp1-1, ade2-1, can1-100, CFIII URA3 SUP11</i>
VAY28 MATa	<i>sfi1-273, ura3-1, leu2,3-112, his3-11, trp1-1, ade2-1, can1-100, CFIII URA3 SUP11</i>
VAY33 MATa	<i>cdc4, TRP1::SPC42-GFP</i>
VAY55 MATa	<i>sfi1-3, TRP1::SPC42-GFP</i>
VAY78 MATa	<i>sfi1-120-mCherry:NAT, TRP1::SPC42-GFP, ura3-1, leu2,3-112, his3-11, trp1-1, ade2-1</i>
VAY87 MATa	<i>SFI1-mCherry:NAT, ura3-1, leu2,3-112, his3-11, trp1-1, ade2-1, can1-100</i>

family. Members of the centrin family have important roles in microtubule-organizing centers such as the SPB, centrosome, and basal bodies; and in higher organisms, they are often the major component of Ca<sup>2+</sup>-sensitive contractile fibers (Schiebel and Bornens, 1995). Sfi1p biochemically interacts with Cdc31p, is localized to the half-bridge of the SPB, and like Cdc31p is required for early steps in SPB duplication (Kilmartin, 2003). Sfi1p contains a central domain composed of multiple, evolutionarily conserved repeats, each of which can bind to Cdc31p (Kilmartin, 2003). It was suggested that Sfi1p-centrin fibers could act as elastic connections between various elements of the centrosome and that they might mediate a licensing step necessary for centrosome duplication (Salisbury, 2004). Recently, the crystal structures of two Sfi1p-centrin complexes were reported, and it was confirmed that multiple molecules of Cdc31p bind a single Sfi1p, resulting in a filamentous structure (Li *et al.*, 2006). On the basis of electron microscopy (EM) evidence placing the N terminus of Sfi1p at the SPB and the C terminus at the center of the bridge, it was proposed that an Sfi1p/Cdc31p filament could span the length of the half-bridge. Kilmartin and coworkers suggested that during SPB duplication, the half-bridge could double in length through association of two, end-to-end Sfi1 C termini, providing a new Sfi1 N terminus as an assembly site for the new SPB (Li *et al.*, 2006). However, these important functions for the N- and C-terminal domains of Sfi1p have yet to be investigated experimentally.

Here, we describe new *sfi1* mutants that arrest in mitosis with duplicated, side-by-side SPBs, indicating that Sfi1p does have further roles to play in addition to its previously described function in centrin-mediated SPB duplication. These mutations all map to the C terminus of Sfi1p, and they are entirely consistent with models in which the Sfi1p C terminus has an important role in separation or splitting of the duplicated SPBs during bipolar spindle assembly.

## MATERIALS AND METHODS

General yeast methods and media were used except where stated (Guthrie *et al.*, 2004). Standard stocks were 10 mg/ml  $\alpha$  factor, 10 mg/ml nocodazole, 100 mg/ml hydroxyurea, and 30 mg/ml benomyl. Yeast transformation and

polymerase chain reaction (PCR)-mediated epitope tagging were carried out using standard procedures (Ito *et al.*, 1983; Longtine *et al.*, 1998), and for mCherry (Shaner *et al.*, 2004; Snaith *et al.*, 2005). DNA sequencing was carried out by PCR according to manufacturer's instructions (ABI Big Dye; Applied Biosystems, Foster City, CA). Table 1 lists the yeast strains used, all of which are derivatives of W303.

### Yeast Colony Sectoring Assays

The quantitative chromosome loss assay (CFIII) was performed as described previously (Warren *et al.*, 2002).

### Linkage Analysis of the Novel *sfi1* Alleles

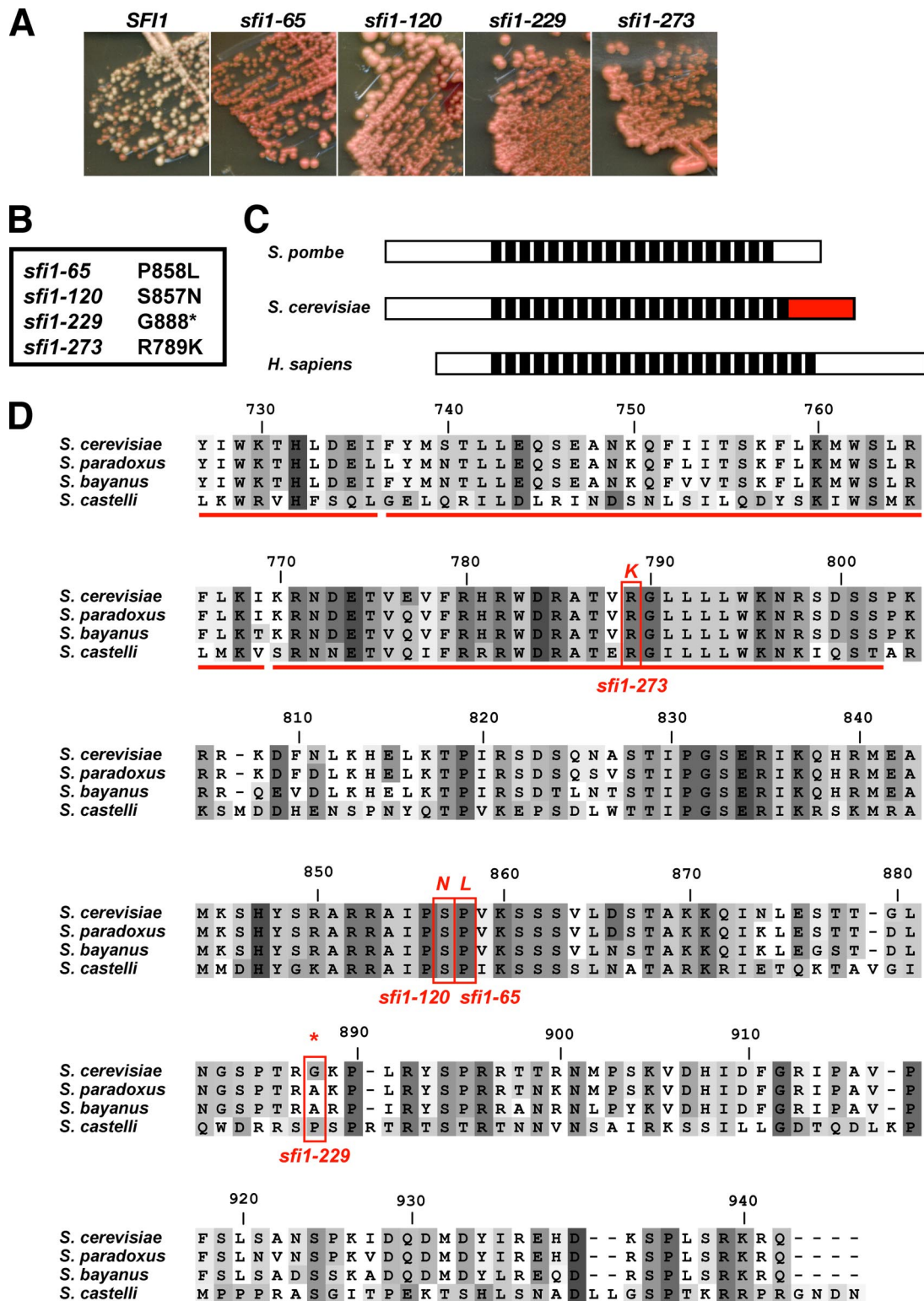
To test genetically whether the synthetic lethal mutations were tightly linked to the *sfi1* locus, we constructed a wild-type *SFI1, mad1Δ* strain in which the *URA3* marker was integrated just upstream of *SFI1*. This strain was then crossed with the synthetic lethal mutants, diploids were sporulated, and tetrads were dissected. If the synthetic lethal mutation was linked to the *SFI1* locus, no spores would be recovered that were *URA3+* and that contained the synthetic lethal mutation. The latter was assessed visually, using the *MAD1, ADE3* plasmid and colony sectoring assay. We dissected at least 24 tetrads for each *sfi1* allele, and we never saw recombination between the mutation and *URA3*. This showed that each mutation was tightly linked to, and most likely lay within, the *SFI1* gene. This was later confirmed by sequencing these *sfi1* alleles (Figure 1B).

### Synthetic Lethality of the *sfi1*-C-Terminal Mutations with *mad3Δ* and *bub1Δ*

The *sfi1* mutations were all crossed with strains containing either a *mad3* or *bub1* deletion that was complemented by a plasmid carrying *MAD3* or *BUB1* and the *URA3* marker. The double mutants isolated from these crosses were able to grow on -URA media but not on plates containing 5-fluoroorotic acid, indicating that the *MAD3* or *BUB1* gene were needed for viability, and that the *sfi1* alleles are also synthetic lethal with other checkpoint mutations.

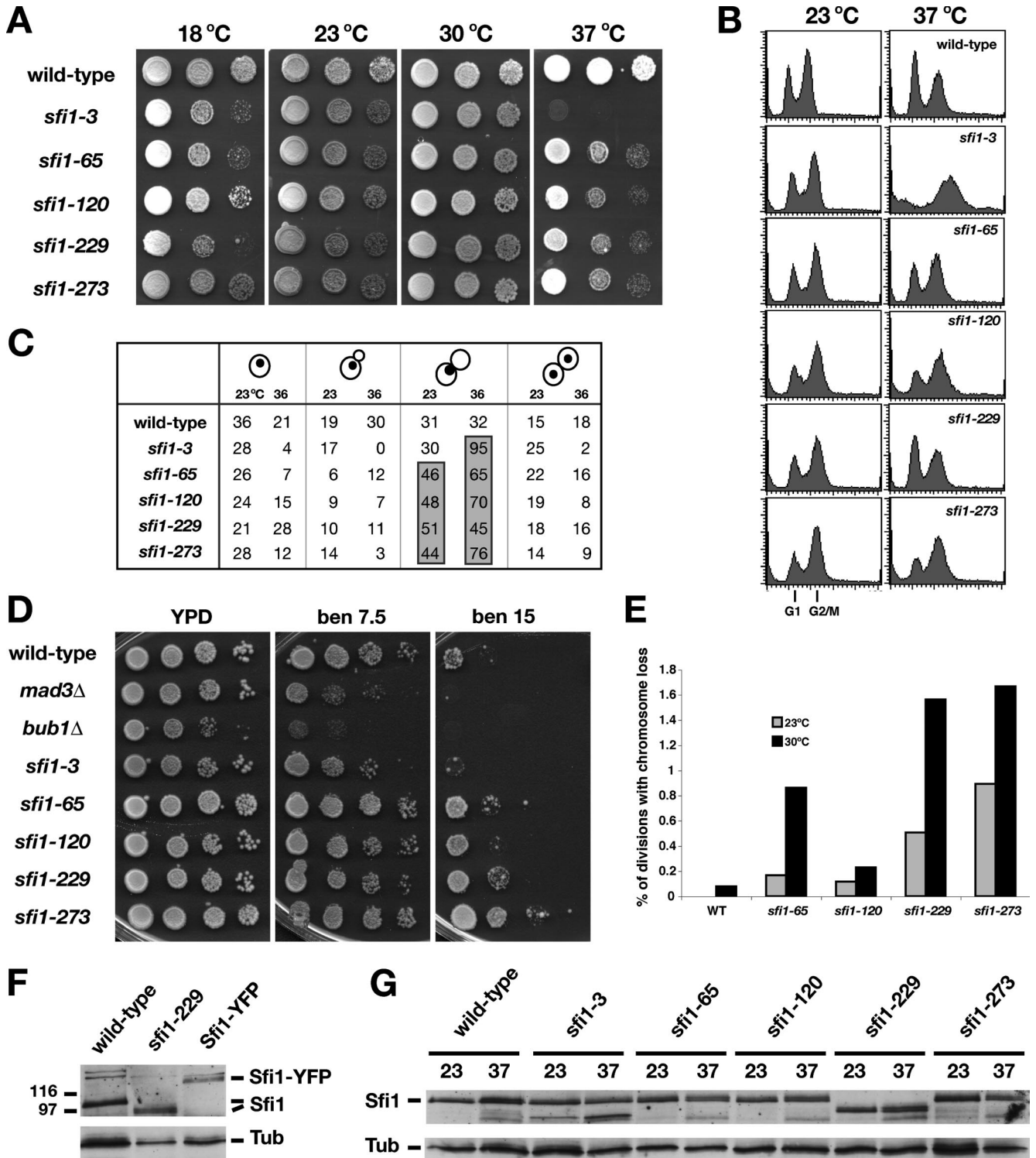
### *Sfi1p* Antibodies

Nucleotides 1-1655 of *SFI1* were PCR amplified and cloned into pGEX-6P. Bacterial cultures were induced with isopropyl  $\beta$ -D-thiogalactoside overnight at 18°C, pelleted, frozen, and then ground in a pestle and mortar under liquid nitrogen. Extracts were thawed in lysis buffer (phosphate-buffered saline, 1 M NaCl, 1 mM phenylmethylsulfonyl fluoride, and 0.5% Tween 20), sonicated for 1 min, and clarified. Sfi1-glutathione S-transferase (GST) fusion protein was then purified on glutathione agarose by using standard protocols, dialyzed overnight (50 mM HEPES, pH 7.6, 100 mM KCl, and 30% glycerol), and used as antigen. Anti-Sfi1p serum was affinity purified on a column of Sfi1-GST coupled to Affigel 10 (Bio-Rad, Hercules, CA) as described previously (Hardwick and Murray, 1995). Immunoblotting was carried out as described previously (Hardwick and Murray, 1995).



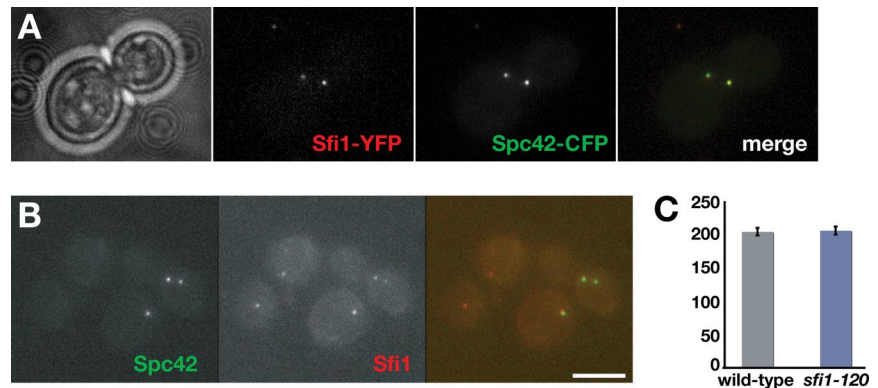
**Figure 1.** The C terminus of Sfi1p is highly conserved among budding yeast, and it is mutated in the novel *mad1*-synthetic lethal *sfi1* alleles. (A) The four mutants (*sfi1-65*, *120*, *229*, and *273*) are synthetically lethal with a *mad1Δ*; therefore, they are unable to grow without the *MAD1*, *ADE3* plasmid. Colonies containing this *ADE3* plasmid were red (as they are *ade2-*), whereas those that lost it were white (*ade2-*, *ade3-*). Only the strain containing wild-type *SFI1* was able to lose the plasmid and form white sectors and colonies. (B) Three of the *sfi1* alleles have mutations in conserved residues, and the fourth encodes a truncated protein. (C) Schematic diagram of the Sfi1 protein indicating the three domains. The central domain consists of ~20 repeats that are conserved from yeast to human. The red region highlights the domain of Sfi1p containing the synthetic lethal mutations. (D) Sequence alignment of the C terminus of Sfi1p from four different *Saccharomyces* species (modified from <http://db.yeastgenome.org/fungi/YLL003W.html>). The four mutations identified in this study are indicated. Red bars underneath the sequence indicate the last three repeats (19–21) from the central domain.





**Figure 2.** The novel *sfi1* alleles are temperature sensitive, resistant to benomyl, and display increased chromosome loss. (A) *sfi1-65*, *sfi1-120*, *sfi1-229*, and *sfi1-273* are temperature and cold sensitive, but far less so than *sfi1-3*. Yeast strains were diluted then spotted onto rich media (YPD) plates. Images were taken after 3-d growth at the indicated temperatures. (B) Wild-type, *sfi1-3*, *sfi1-65*, *sfi1-120*, *sfi1-229*, and *sfi1-273* cultures were shifted to 37°C for 4 h, fixed, and processed for FACS analysis ( $n = 20,000$  cells). Although *sfi1-3* clearly arrested with a G2/M DNA content (74% of cells), this was less apparent for the other *sfi1* alleles (62, 63, 53, and 66% respectively), which is consistent with them having a less penetrant arrest. (C) Cells from B were analyzed by light and fluorescence microscopy (DNA was stained with propidium iodide during the FACS protocol), and the budding index of the various cultures was scored. The four novel *sfi1* alleles all displayed an accumulation of large-budded cells, and with the exception of *sfi1-229*, this was enhanced at 37°C. (D) The novel *sfi1* alleles are benomyl resistant. The yeast strains were diluted and spotted onto YPD plates containing the indicated concentration of the antimicrotubule drug benomyl (micrograms per milliliter). Images were taken after 3-d growth at 24°C. (E) A colony sectoring assay (CFIII) was used to measure the chromosome loss rates of the novel *sfi1* alleles at 23 and 30°C. Numbers indicate the percentage of divisions leading to loss of the marker chromosome during

**Figure 3.** The C-terminal mutation of Sfi1p does not affect its targeting to the yeast SPB. (A) Wild-type Sfi1-YFP colocalizes with the spindle pole body marker Spc42-CFP. Cells were fixed briefly for 5 min in 3.7% formaldehyde, and then they were imaged using a CFP/YFP filter set. (B) Cells containing Sfi1-mCherry, or *sfi1-120*-mCherry and Spc42-GFP, were fixed briefly for 5 min in 3.7% formaldehyde, and then they were mixed and imaged together. Spc42-GFP was used to distinguish the two cell types. The mutant Sfi1 protein was found at SPBs. Bar, 5  $\mu$ m. (C) Quantitation of images from these same mixed cultures showing that both wild-type and the *sfi1-120* mutant protein were found at SPBs at very similar levels. The y-axis shows mean pixel intensity at the SPBs, in arbitrary units, as measured in Slidebook. We analyzed 149 *sfi1-120* cells and 270 wild-type cells. Bars indicate SD.



### Light Microscopy

Strains were grown in complete synthetic media (CSM) or the appropriate selective CSM media (Qbiogene, Carlsbad, CA) made up to double recommended concentration. For GFP/YFP/CFP/mCherry imaging, cells were analyzed live, or they fixed briefly with formaldehyde (typically 3.7% for 5–10 min). Microscopy was performed with a Zeiss Axiovert 200 Marianas inverted microscope system from Intelligent Imaging Innovations (Denver, CO), with a CoolSNAP HQ charge-coupled device camera. Images were taken and analyzed using Slidebook software (Intelligent Imaging Innovations).

### Electron Microscopy

Cells were fixed, processed, and imaged as described previously (Giddings *et al.*, 2001). Twenty-four *sfi1* cells were analyzed very thoroughly by serial section.

## RESULTS

### Isolation of Novel Alleles of *sfi1*

A *mad1* synthetic lethal screen (Hardwick, unpublished data) isolated four novel *sfi1* alleles. The screen used an *ADE3*, *MAD1* plasmid sectoring assay and *ade2*, *ade3*, *mad1* $\Delta$  mutants. These strains are red if they maintain the *ADE3* plasmid, but they are white if they lose it. The synthetic lethal mutants die if they lose the *MAD1*, *ADE3* plasmid and therefore grow to form entirely red colonies (Figure 1A). These four mutants were all rescued with an *SFI1* clone from a YCP50-based genomic library (Hardwick and Murray, 1995), enabling them to lose the *MAD1*, *ADE3* plasmid and form sectored colonies (data not shown, but see Figure 6). Linkage experiments suggested that the synthetic lethal mutations were likely to be *sfi1* alleles rather than being suppressed by the *SFI1* gene (see *Materials and Methods* for details). The *sfi1* locus was then PCR amplified using genomic DNA from all four strains, sequenced, and found to contain mutations in, or very close to, the C-terminal domain of Sfi1p (Figure 1B). This domain lies after the central repeat domain that has been shown to interact with Cdc31p. The *sfi1-273* allele (R789K) actually lies in the last of the 21 repeats thought to interact with centrin (Li *et al.*, 2006), but it could perturb the overall structure of the C terminus. Al-

though the C terminus is not conserved in other model organisms or human Sfi1p, database analysis showed that it, if the last repeat is included, is the section of Sfi1p with the highest levels of homology among budding yeast (ranging from 40% identity for *Saccharomyces cerevisiae* and *Saccharomyces castellii* to 98% identity for *S. cerevisiae* and *S. paradoxus*; Figure 1C). This suggested that the C terminus of Sfi1p has an important conserved function, at least in budding yeast.

Their isolation as *mad1* synthetic lethal mutations suggested that these *sfi1* alleles have mitotic defects, even at 23°C, because they are dependent on the spindle checkpoint for viability. To further our understanding of their phenotypes, we analyzed the growth of these *sfi1* alleles in different conditions, and we compared them with the tight temperature-sensitive *sfi1-3* allele (this contains mutations H207Q, F208C, Y247C; Kilmartin, 2003). The new *sfi1* mutants grew significantly slower than wild type at 18 and 36°C, although their cold-sensitive phenotype was somewhat variable. We analyzed their temperature sensitivity further by shifting log phase cultures to 36°C for 4 h and then analyzing cells by fluorescence-activated cell sorting and microscopy. No clear DNA replication defect was observed by FACS (Figure 2B), but a significant increase in the number of large-budded cells was observed in all four strains (Figure 2C). Such an accumulation of large-budded cells with 2C DNA is consistent with a mitotic delay due to spindle checkpoint activation. Next, we tested whether these *sfi1* alleles were sensitive to antimicrotubule drugs, and we found them to be benomyl resistant (Figure 2D). This is a relatively unusual phenotype and suggests the possibility that these mutants somehow stabilize microtubules, thereby counteracting the effects of antimicrotubule drugs.

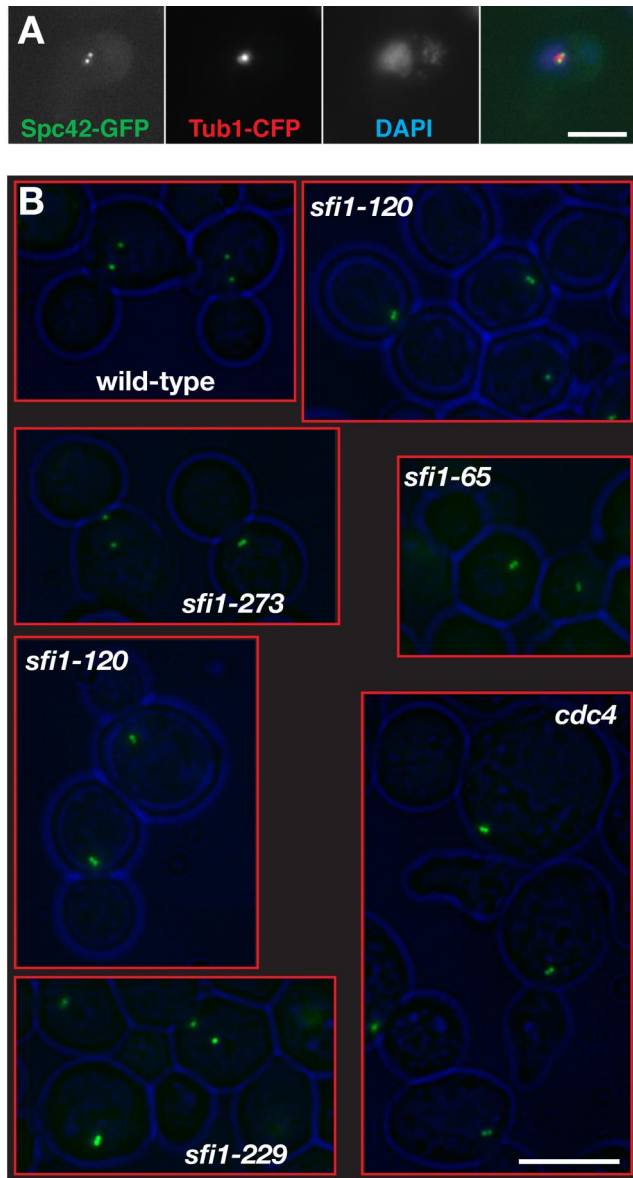
Mitotic defects in budding yeast tend to lead to an increased chromosome loss rate. To quantitate this in these *sfi1* alleles, we used another colony sectoring assay (see *Materials and Methods* for details). All four *sfi1* alleles demonstrated increased chromosome loss at semipermissive temperatures (30°C), and they all showed subtle defects at 23°C (Figure 2C). The latter result was to be expected, because even at 23°C these mutations require the spindle checkpoint for viability. Note, these *sfi1* mutations are also synthetic lethal with *mad3* $\Delta$  and *bub1* $\Delta$  (see *Materials and Methods* for details), indicating that this is not a genetic interaction that is specific to the loss of Mad1p function.

### Protein Stability and Localization of Sfi1p Are Unaffected

Next, we analyzed the mutant Sfi1 proteins, to determine whether their stability was affected upon temperature shift.

**Figure 2 (cont).** the first division on the solid media (half-sector). (F) Immunoblots of total protein extracts using anti-Sfi1p antibody. Specificity of the antibody is shown by the gel shifts in Sfi1p found in the *sfi1-229* truncation allele and a strain where the wild-type protein had been tagged with YFP. (G) All mutant alleles of *sfi1* express stable proteins. Yeast cultures were grown at 23 or 37°C for 3 h, total protein extracts were made, and they were immunoblotted for Sfi1p.





**Figure 4.** The *sfi1* C-terminal mutants have duplicated SPBs but abnormally short spindles. (A) *sfi1-65* cells containing Spc42-GFP and Tub1-CFP were shifted to 37°C for 3 h, fixed briefly for 5 min in 3.7% formaldehyde, and then they were imaged to analyze SPB

To do this, we raised polyclonal antibodies to the N terminus of Sfi1p (see *Materials and Methods*), and we used these on immunoblots of total protein extracts. These antibodies recognized wild-type Sfi1p, which ran at the predicted size of 112 kDa. As expected, a truncated protein was detected in *sfi1-229* (97 kDa), and a larger fusion protein of ~140 kDa was recognized in the strain expressing Sfi1-YFP (Figure 2F). We immunoblotted extracts made from wild-type and *sfi1* mutant cells grown at 23 and 37°C. This showed that all of the mutant Sfi1 proteins were stable, even at 37°C (Figure 2G). We conclude that the C-terminal *sfi1* mutations do not significantly affect the stability of the Sfi1 protein.

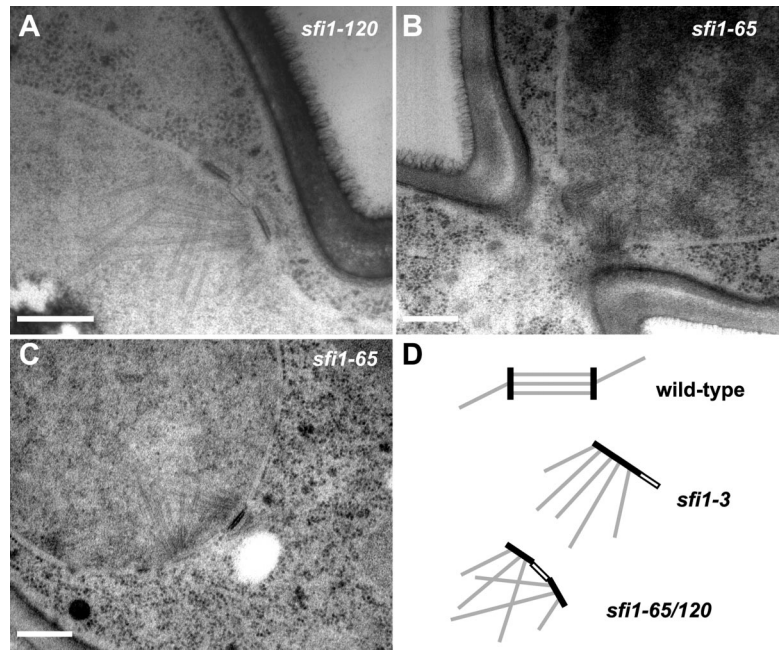
We used double-label fluorescence experiments to confirm that Sfi1p localizes to budding yeast SPBs (Figure 3A; Kilmartin, 2003). To determine whether our *mad1* synthetic lethal *sfi1* alleles were defective in SPB targeting, we fused the monomeric red fluorescent protein mCherry (Shaner *et al.*, 2004) to *sfi1-120*. To ensure identical exposure times and image processing, we imaged a mixture of mCherry-tagged *sfi1-120p* mutant cells, which also expressed Spc42-green fluorescent protein (GFP), and mCherry-tagged wild-type Sfi1p cells that did not express Spc42-GFP. The *sfi1-120* mutant protein was still detected at SPBs (Figure 3B), and quantitative analysis showed that it was there at wild-type levels (Figure 3C). Thus, the *sfi1* C-terminal point mutants do not seem to affect the stability of Sfi1p or its targeting to the SPB.

#### Analysis of SPBs and Spindles in *sfi1* Mutants

*sfi1-3* (H207Q, F208C, Y247C) and *sfi1-7* (N218D, D242A) are tight conditional (ts) alleles that arrest with an elongated but unduplicated SPB (Kilmartin, 2003). Analysis of spindles in *sfi1-65* and *sfi1-120* alleles revealed a very distinct phenotype (Figure 4). We presynchronized cells in G1 with  $\alpha$ -factor, and then we released them to 37°C for 3 h, before fixing them briefly with formaldehyde. When analyzed by light microscopy, using cyan fluorescent protein (CFP)-tubulin and Spc42-GFP, we observed a significant enrichment of cells with paired GFP spots (Figure 4, A and B). The two Spc42-GFP spots were typically ~0.3  $\mu\text{m}$  apart. These spindle poles were much closer together than in a normal short mitotic spindle (~1.2  $\mu\text{m}$  in early wild-type mitosis), and they had a very similar separation to the spindles observed in *cdc4* mutants (also 0.3  $\mu\text{m}$ ), which are known to arrest with duplicated but unseparated SPBs (Figure 4C; Byers and Goetsch, 1974). This suggested that the new *sfi1* alleles had arrested with side-by-side SPBs or with very short bipolar spindles.

To look at these spindle defects in more detail, we used thin-section electron microscopy, and we analyzed serial sections. *sfi1-65* and *sfi1-120* cells were  $\alpha$ -factor arrested and then released at 37°C for 3 h before high-pressure freezing. Although some large-budded cells had bipolar spindles that looked normal, 50% had duplicated side-by-side SPBs (Fig-

separation and spindle structures. (B) Cells were presynchronized cells in G1 with  $\alpha$ -factor, released at 37°C for 3 h, and then fixed briefly with formaldehyde. These strains contain Spc42-GFP, and they were imaged to look at their SPB number and position. Bar, 5  $\mu\text{m}$ . (C) Quantitation of the distances separating the SPBs in the aforementioned strains. *sfi1-65*, *sfi1-120*, *sfi1-229*, and *sfi1-273* all displayed increased numbers of short (<0.6- $\mu\text{m}$ ) spindles, which were very similar in appearance to the *cdc4* control strain. A minimum of 118 cells were counted per sample (n = 444 for wild type and 380 for *sfi1-65*, 312 for *sfi1-120*, 283 for *sfi1-229*, 118 for *sfi1-273*, and 167 for *cdc4*).



**Figure 5.** Electron microscopy reveals duplicated but unseparated SPBs in the *sfi1* C-terminal mutants. Cells were presynchronized cells in G1 with  $\alpha$ -factor and released at 37°C for 3 h. Cells were high-pressure frozen, and then they were freeze-substituted in 2% osmium tetroxide and 0.1% uranyl acetate (see Giddings *et al.*, 2001 for further details). (A) EM image of side-by-side SPBs in *sfi1-120*. (B and C) EM images of SPBs in *sfi1-65*. Bars are 0.3  $\mu$ m. (D) Schematic representation of the SPB phenotype observed by EM, in different *sfi1* alleles. Note, this is our interpretation of the *sfi1-3* phenotype from data in Kilmartin (2003).

ure 5). These SPBs were of equal and normal size, had a clear half-bridge connecting them, and had nuclear microtubules emanating from them. This demonstrates that the *sfi1-65* and *sfi1-120* defect is postduplication and that it does not affect the ability of the SPBs to nucleate microtubules. Thus, these *sfi1* mutants have a defect in SPB separation. This analysis by electron microscopy demonstrates that Sfi1p has an additional role to play in bipolar spindle formation after SPB duplication.

#### Genetic Interactions of *sfi1* C-Terminal Mutants

The spindle defect in the *sfi1* C-terminal mutants is quite distinct from that of the previously described *sfi1-3* and *sfi1-7* alleles. The *sfi1-3* and *sfi1-7* defect is probably due to impaired interaction with the budding yeast centrin protein Cdc31p, and their temperature sensitivity could be suppressed by overexpression of *CDC31* (Kilmartin, 2003). To confirm that the *sfi1* C-terminal defect(s) of mutants was mechanistically distinct from that of *sfi1-3* and *sfi1-7*, we tested whether *CDC31* overexpression could also suppress their temperature sensitivity. This was not the case (Figure 6A). We next tested a bank of high copy (2- $\mu$ m) plasmids encoding other SPB components to see whether any of them could suppress the *sfi1* C-terminal alleles. To do this, we tested whether their overexpression could suppress the synthetic lethality of the *sfi1* C-terminal mutant alleles with *mad1 $\Delta$* , by using the *MAD1*, *ADE3* plasmid and colony sectoring assay. These *sfi1* mutants require *MAD1* for viability, and by maintaining the *MAD1*, *ADE3* plasmid, they form red colonies (because they are chromosomally *ade2*, *ade3* mutants). If the 2- $\mu$ m plasmid suppresses the *sfi1* mutation, they are then able to lose the *ADE3* plasmid and form red/white sector colonies (Figure 6B). As expected, plasmids encoding either *SFI1* or *MAD1* allowed frequent colony sectoring. In addition, we found that *BBP1*, *NDC1*, *MPS2*, *KAR1*, and *CIN8* plasmids could all suppress the synthetic lethality to different extents (Figure 6C). Bbp1, Mps2, and Ndc1 are all implicated in the process of inserting the newly formed SPB duplication plaque into the nuclear membrane, and they possibly have roles in anchoring the

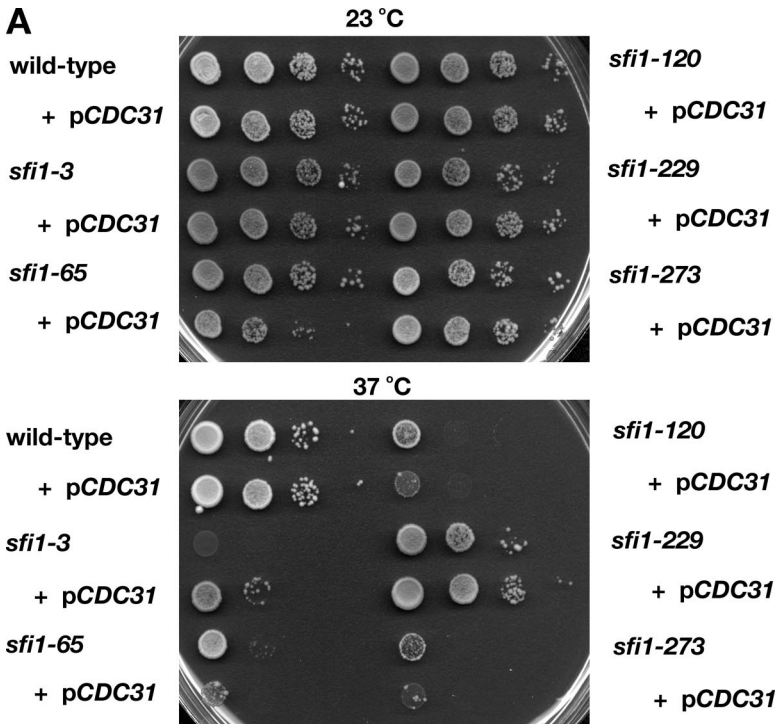
SPB once inserted (Chial *et al.*, 1998; Schramm *et al.*, 2000; Park *et al.*, 2004; Araki *et al.*, 2006). This suggests that at least part of the *sfi1* C-terminal mutant phenotype could reflect a defective interaction between the SPB and the nuclear envelope. However, our EM analysis does not support an insertion defect, because the duplicated SPBs seem to be sitting normally in the nuclear envelope, and they are able to nucleate microtubules. It is not known what other roles the Mps2/Bbp1 membrane protein complex has to play in spindle biogenesis.

#### DISCUSSION

We have isolated several novel *sfi1* alleles and shown by light and electron microscopy that they have phenotypes distinct from those described previously for other ts alleles of *sfi1*. This was confirmed through analysis of genetic interactions with *CDC31*. These findings demonstrate that Sfi1p is involved in aspects of bipolar spindle biogenesis after the SPBs have been duplicated. The electron microscopy suggests that the novel *sfi1* defect is in separation of the duplicated SPBs, possibly defective splitting of the bridge.

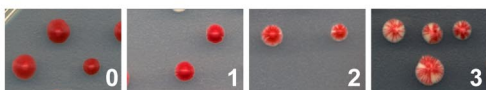
#### Multiple Functions for Sfi1p

Sfi1p had already been shown to have a role in the initial stage of SPB duplication. The *sfi1-3* and *sfi1-7* alleles, which have mutations in the Cdc31p binding repeats, arrest with only one spindle pole body before satellite assembly (Kilmartin, 2003). Recent work from the Kilmartin laboratory has described the crystal structure of Sfi1p-Cdc31p complexes (Li *et al.*, 2006). In addition, it was shown by EM shadowing that Sfi1p-Cdc31p complexes form filaments in isolation. The authors proposed an elegant model describing the role of Sfi1p in SPB duplication. The model has Sfi1p molecules spanning the half-bridge with their N termini anchored in the spindle pole body and their C termini at the distal end. They suggested that upon initiation of SPB duplication additional Sfi1p molecules are recruited to the half-bridge and interact with the existing half-bridge Sfi1p molecules, end on, via their C termini. This end-on interac-



**B**

	<i>sfi1-65</i>	<i>sfi1-120</i>	<i>sfi1-229</i>	<i>sfi1-273</i>
vector	0	0	0	0
p(MAD1)	3	3	3	3
p(SFI1)	2-3	2	2-3	2-3
p(BBP1)	1	0	1	1
p(CDC31)	0	0	0	0
p(CIN8)	0	0	2	2
p(CNM67)	0	0	0	0
p(HSF1)	0	0	0	0
p(KAR1)	0	0	1	0
p(KAR3)	0	0	0	0
p(MPS1)	0	0	0	0
p(MPS2)	0	0	0	1
p(MPS3)	0	0	0	0
p(NBP1)	0	0	0	0
p(NDC1)	2	0-1	2	2
p(NUD1)	0	0	0	0
p(SPC29)	0	0	0	0
p(SPC42)	0	0	0	0
p(SPC98)	0	0	0	0
p(TUB1)	0	0	0	0

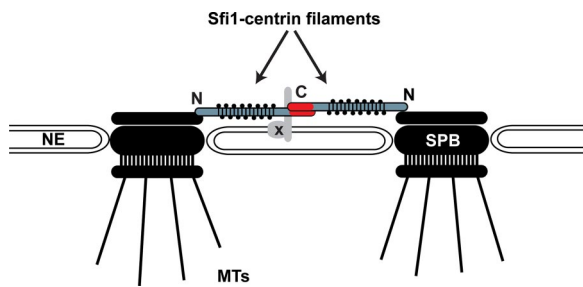


**Figure 6.** The *sfi1* C-terminal mutants are not suppressed by overexpression of *CDC31*, but they do display genetic interactions with other SPB components. (A) The strains indicated, either containing or lacking extra plasmid-borne copies of the *CDC31* gene, were diluted and spotted onto YPD plates. Images were taken after 3-d growth at either 23 or 37°C. Only *sfi1-3* was suppressed, and *sfi1-65*, *sfi1-120*, and *sfi1-273* all grew slower in the presence of extra *CDC31*. (B) The four *sfi1*, *mad1Δ* alleles were all transformed with the bank of plasmids listed in the left column. The ability of the transformant to sector was scored on the scale 0 (no sectoring, red colonies) to 3 (considerable sectoring with large white sectors). Sectoring indicates that the strain had been able to lose a *MAD1*, *ADE3* plasmid. This could be due to either rescue of the *mad1* or *sfi1* mutation (as in rows 2 and 3) or suppression of the *sfi1* mutant phenotype by the other plasmids.

tion results in a doubling of the bridge length, and the Sfi1 C termini are now positioned at the center of the bridge (Figure 7). This model was strongly supported by immuno-EM analysis of strains that expressed Sfi1p with either N- or C-terminal GFP tags: the N terminus was positioned next to the junction between the SPB and the proximal end of the half-bridge, whereas the C-terminal tag was found to be close to the distal end, or in the center of the bridge in paired SPBs (Li *et al.*, 2006). Thus, the Sfi1p C termini are ideally placed to mediate interactions necessary for bridge separation. It was suggested that an important role for the Sfi1p C terminus might be in mediating the cleavage process, either alone or in combination with other proteins.

Our findings provide very strong support for such a model. In this study, we have isolated novel alleles of *sfi1* that arrest late in spindle pole body duplication, with duplicated but unseparated SPBs. Our EM analysis of these *sfi1* alleles is consistent with a defect in bridge cleavage, and the mutations conferring this phenotype all mapped to the C terminus of Sfi1p. To develop this model further, it will be important to find proteins that interact with the C terminus of Sfi1p. In an attempt to do this, we carried out two-hybrid screens and Sfi1p affinity chromatography (with C-terminal Sfi1p-GST fusion proteins), but as yet we have failed to find any interactors (data not shown). One possible explanation for this failure is that the interacting Sfi1p partners could be





**Figure 7.** Model of Sfi1 function during SPB duplication and splitting. Schematic representation of the Sfi1-centrin filament spanning the bridge of the duplicated SPBs. Factor X is predicted to interact with the C terminus of Sfi1p and to be instrumental in the cleavage of the bridge upon bipolar spindle formation.

integral membrane proteins, which would be difficult to identify with either of the approaches taken.

Our genetic studies confirm that the *sfi1* C-terminal mutant phenotype is novel and shows that it is not due to a defective interaction with Cdc31p, as was found for the *sfi1-3* and *sfi1-7* alleles. In addition, our multicopy suppression analysis identified other components of the spindle pole body that can partially rescue the defect of *sfi1* C-terminal mutations. One of these components was the kinesin motor protein Cin8p. This is a plus-end-directed motor required to separate duplicated SPBs by producing an outward force on the bipolar spindle (Saunders and Hoyt, 1992; Saunders *et al.*, 1997). We suggest that overexpression of the Cin8 motor protein might increase this outwards force to such an extent that it is capable of splitting and driving apart the duplicated SPBs in the *sfi1-229* and *sfi1-273* mutants.

Some of the *sfi1* suppressors encode integral membrane proteins (Ndc1p, Mps2p) of the nuclear envelope, and others are known to form a complex with them (Mps2-Bbp1-Nbp1). The terminal phenotype of conditional alleles of these SPB components is typically a “dead” SPB, which fails to nucleate nuclear microtubules (Chial *et al.*, 1998; Schramm *et al.*, 2000; Park *et al.*, 2004; Araki *et al.*, 2006). This is due to a failure to insert the new SPB into the nuclear envelope. Could these complexes interact with the Sfi1p C termini? It was recently shown that bridge components, such as Sfi1p, Cdc31p, Mps3p, and Kar1p, are all present on these dead SPBs (Araki *et al.*, 2006), so it is unlikely that these factors are involved in targeting or maintaining Sfi1p at the bridge. Whether the “insertion” factors have later roles to play in spindle biogenesis is unclear, although it is thought that Nbp1 is required to maintain Mps2p at the SPB (Araki *et al.*, 2006). We propose that the C termini of Sfi1p do have an important role to play in bridge splitting, as proposed originally (Li *et al.*, 2006), and that this separation event might require interaction with integral components of the nuclear envelope, such as the Mps2-Bbp1 complex (see model in Figure 7).

Almost nothing is known about separation of the SPB bridge. Another mutant that arrests at a similar stage is *cdc4*, an F-box component of the ubiquitin ligase known as the SCF (Mathias *et al.*, 1996; Cardozo and Pagano, 2004). This suggests the possibility that an SCF substrate needs to be degraded, by ubiquitin-mediated proteolysis, for bridge separation to occur. It is tempting to think that a half-bridge linker molecule is itself the SCF substrate, but it is also possible that the SCF substrate regulates bridge separation. Indeed, SCF activity may simply be needed to get to the correct stage of the cell cycle for SPB separation to occur,

perhaps through the activation of microtubule motors and changes in microtubule dynamics after S phase onset. That increased Cin8 motor protein expression can suppress two of the *sfi1* alleles is interesting and suggests that increased motor activity can be sufficient to drive defective Sfi1p C termini apart leading to SPB separation.

It will be of interest to discover whether Sfi1 homologues have roles to play in splitting and separation of vertebrate centrosomes, and if so, whether the Sfi1 proteins are perturbed in cancers. Many human tumors display centrosome aberrations, but it is unclear as to whether these are typically the cause or consequence of cancer progression (Nigg, 2002). Either way, it is possible that the spindle checkpoint could make a useful drug target for anticancer therapy, because we have shown here that the combined loss of Sfi1p and Mad1p functions can be completely lethal to cells.

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