Comprehensive Genetic Analysis of Paralogous Terminal Septin Subunits Shs1 and Cdc11 in *Saccharomyces cerevisiae*

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ABSTRACT Septins are a family of GTP-binding proteins considered to be cytoskeletal elements because they self-assemble into filaments and other higher-order structures *in vivo*. In budding yeast, septins establish a diffusion barrier at the bud neck between a mother and daughter cell, promote membrane curvature there, and serve as a scaffold to recruit other proteins to the site of cytokinesis. However, the mechanism by which any septin engages a partner protein has been unclear. The two most related and recently evolved subunits appear to be Cdc11 and Shs1, and the basic building blocks for assembling septin structures are hetero-octameric rods (Cdc11–Cdc12–Cdc3–Cdc10–Cdc10–Cdc3–Cdc12–Cdc11 and Shs1–Cdc12–Cdc3–Cdc10–Cdc10–Cdc3–Cdc12–Shs1). Loss of Cdc11 is not normally tolerated, whereas cells lacking Shs1 do not appear grossly abnormal. We established several different sensitized genetic backgrounds wherein Shs1 is indispensable, which allowed us to carry out the first comprehensive and detailed genetic analysis of Shs1 *in vivo*. Our analysis revealed several novel insights, including: (i) the sole portion of Shs1 essential for its function is a predicted coiled-coil-forming segment in its C-terminal extension (CTE); (ii) the CTE of Cdc11 shares this function; (iii) this role for the CTEs of Cdc11 and Shs1 is quite distinct from that of the CTEs of Cdc3 and Cdc12; and (iv) heterotypic Cdc11 and Shs1 junctions likely occur *in vivo*.

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SEPTINS are GTP-binding proteins conserved across *Eukarya* (except higher plants) (Pan *et al.* 2007; Nishihama *et al.* 2011). This protein family was first identified because the corresponding loci were among the temperature-sensitive (ts) cell division cycle (*cdc*) mutations isolated in *Saccharomyces cerevisiae* (Hartwell 1978). At the restrictive temperature, *cdc3*, *cdc10*, *cdc11*, and *cdc12* mutants continued to bud, replicate, and segregate their chromosomes, yet failed to execute cell division, resulting in the formation of chains of multinucleate cells (Hartwell 1971). Shifting a ts allele of any of these four homologous gene products to restrictive temperature pre-

vented formation of what appeared to be rings of highly ordered membrane-associated filaments at the bud neck (Byers and Goetsch 1976). Antibody decoration (Haarer and Pringle 1987; Ford and Pringle 1991; Kim *et al.* 1991) and, later, use of fusions to green fluorescent protein (GFP) (Cid *et al.* 1998) demonstrated that these four proteins colocalized with and were likely constituents of these filaments. Because loss of the function of these proteins prevents cytokinesis and cell septation, and they seemed to be integral components of the filamentous structures erected at the bud neck, they were dubbed septins (Sanders and Field 1994; Pringle 2008). Indeed, subsequent purification of these proteins from yeast (Frazier *et al.* 1998), and their production as recombinant proteins in bacteria (Versele *et al.* 2004; Versele and Thorner 2004; Farkasovsky *et al.* 2005), demonstrated that Cdc3, Cdc10, Cdc11, and Cdc12 were necessary and sufficient for the formation of long-paired filaments *in vitro* (Bertin *et al.* 2008) that closely resemble those observed at

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the bud neck *in vivo* (Bertin *et al.* 2012; Ong *et al.* 2014). Detailed ultrastructural analysis led to the discovery that the septin complex that is the fundamental building block of the yeast filaments is a linear, hetero-octameric rod, with the order Cdc11–Cdc12–Cdc3–Cdc10–Cdc10–Cdc3–Cdc12–Cdc11 (Bertin *et al.* 2008), which polymerizes end-on-end via Cdc11–Cdc11 interaction. Subsequently, the nonpolar, linear hetero-octamer with twofold rotational symmetry was found to be characteristic of mammalian septin complexes too (Mostowy and Cossart 2012; Fung *et al.* 2014). Each septin subunit comprises a Ras-related GTP-binding domain (Sirajuddin *et al.* 2007) preceded by an N-terminal extension of variable length and followed by a C-terminal extension (CTE) of variable length (Versele *et al.* 2004; Weirich *et al.* 2008).

However, when the sequence of the entire *S. cerevisiae* genome was determined (Mewes *et al.* 1997), it was revealed that three other genes highly related to *CDC3*, *CDC10*, *CDC11*, and *CDC12* were present. Two of them, *SPR3* and *SPR28*, encode proteins expressed exclusively in *MATa*/*MATα* cells and only under conditions that induce meiosis and sporulation (Neiman 2011; Brar *et al.* 2012). The third, *SHS1/SEP7* (Carroll *et al.* 1998; Mino *et al.* 1998), encodes a protein expressed in mitotically growing haploids and diploids, but, unlike loss of *Cdc3*, *Cdc10*, *Cdc11*, or *Cdc12*, even the complete absence of *Shs1* seemed to have only a very modest effect on cell growth and morphology (Iwase *et al.* 2007; Garcia *et al.* 2011). Biochemical and ultrastructural analysis demonstrated that *Shs1* occupies the same terminal position in the linear hetero-octamer as *Cdc11*, and that such *Shs1*–*Cdc12*–*Cdc3*–*Cdc10*–*Cdc10*–*Cdc3*–*Cdc12*–*Shs1* rods do not polymerize into long-paired filaments *in vitro*, but rather assemble into much more elaborate structures (rings, spirals, and gauzes, depending on the conditions) (Garcia *et al.* 2011). Nonetheless, these findings left open the question of what the two alternative terminal subunits, *Cdc11* and *Shs1*, each contribute to the supramolecular architecture of septin structures and to the physiological function of those structures *in vivo*. Titration of *Shs1*-capped hetero-octamers with increasing amounts of *Cdc11*-capped hetero-octamers reduces the thickness and increases the diameter of the rings formed *in vitro* (Garcia *et al.* 2011). Consistent with a role for *Shs1*-capped rods in modulating the plasticity of septin-based structures, the *Shs1* ortholog in *Candida albicans* contributes to septin ring dynamics (Gonzalez-Novo *et al.* 2008) and the *Shs1* ortholog in *Ashbya gossypii* has been implicated in scaling the size of the septin ring *in vivo* (Meseroll *et al.* 2012). Moreover, in keeping with a primarily regulatory role, *Shs1* undergoes extensive post-translational modification during passage through the cell cycle, including SUMOylation (Johnson and Blobel 1999) and phosphorylation (Mortensen *et al.* 2002; Dobbelaere *et al.* 2003; Smolka *et al.* 2006; Egelhofer *et al.* 2008).

In contrast to cells lacking *Shs1*, cells lacking *Cdc11* either are, depending on strain background, inviable (Versele *et al.* 2004) or exhibit extremely slow growth and a highly elongated morphology (Frazier *et al.* 1998). Viable *cdc11Δ* cells propagate somewhat better on galactose medium than on

glucose medium for reasons that are not understood (McMurray *et al.* 2011). Remarkably, however, in a strain background where cells lacking *Cdc11* are dead, *cdc11Δ shs1Δ* double mutants are viable and, to survive, require that the resulting heterohexamers polymerize via a nonnative *Cdc12*–*Cdc12* interaction (McMurray *et al.* 2011). Conversely, in a strain background where *cdc11Δ* cells are able to propagate, overexpression of *SHS1* kills them, but not otherwise isogenic *CDC11*⁺ cells (Iwase *et al.* 2007). These results indicate that *Shs1*-capped hetero-octamers alone are not sufficient for viability and, when no competing *Cdc11* is present, *Shs1* caps the *Cdc12* subunit and prevents polymerization of *Cdc12*–*Cdc3*–*Cdc10*–*Cdc10*–*Cdc3*–*Cdc12* complexes, in agreement with the observation that *Shs1*-capped rods are unable to assemble into filaments *in vitro* (Garcia *et al.* 2011). Taken together, these results indicate that the ability to form filaments is needed for the execution of some essential function(s) mediated by the septins (McMurray *et al.* 2011).

Thus, although *Cdc11* and *Shs1* occupy the same terminal position, share greater similarity to each other than do any other pair of *S. cerevisiae* septin subunits as judged by pairwise sequence comparisons, and seem to have evolved relatively recently from a common ancestral gene (Pan *et al.* 2007), they appear to have diverged in certain aspects of their function. As one approach to discern both the unique and shared functions of *Cdc11* and *Shs1*, especially *in vivo*, genetic analysis is a potentially useful tool. However, until recently, and regardless of strain background, loss of *Shs1* has not evoked a readily discernible phenotype that would aid in carrying out such an analysis. As described here, we have now established several different conditions under which absence of *Shs1* is lethal, which allowed us to carry out extensive mutational dissection of this gene product. In this way, we were able to pinpoint previously uncharacterized elements in this septin that are required for its function and to compare their roles to those of the corresponding elements in *Cdc3*, *Cdc10*, *Cdc11*, and *Cdc12*.

Materials and Methods

Yeast strains and plasmids

S. cerevisiae strains used in this study are listed in Table 1. Standard molecular biology techniques were used for recombinant DNA manipulations (Sambrook and Russell 2001). Unless otherwise noted, all constructs were generated, as previously described (Finnigan *et al.* 2011), by using *in vivo* ligation and homologous recombination to generate plasmids of interest from which cassettes could be excised and then used for one-step integration at the endogenous genomic locus (replacing, for essential genes, a chromosomal deletion allele covered by a complementing plasmid). The general strategy applied was, first, to digest a *CEN*-based vector (*e.g.*, pRS315 was most commonly used for this purpose) containing a cloned promoter of interest. Next, the gene of interest (or a segment thereof), a sequence encoding a fluorescent tag (when needed) (Sheff and Thorn 2004), the *ADHI* terminator sequence (Bennetzen and Hall 1982), and a drug resistance marker (Goldstein and McCusker 1999), were amplified by

PCR using synthetic oligonucleotides containing tails with sequence homology to each of the fragments so as to assemble them in the desired order and to ensure their insertion downstream of the plasmid-borne promoter. The resulting linear PCR product and the gapped plasmid were then introduced by DNA-mediated transformation using a modified lithium acetate procedure (Eckert-Boulet *et al.* 2012) into appropriate yeast recipients, THS4218 (SF838–1D α *HIS4 his3 Δ ::Hyg^R*) or THS4213 (SF838–1D α *HIS4*), and selected on an appropriate drop-out or drug-containing medium. The resulting plasmids were rescued from yeast, propagated in *Escherichia coli* TOP10 (Lucigen Corp., Middleton, WI), purified (Sambrook and Russell 2001), and confirmed via automated DNA sequence analysis conducted by the University of California (UC) Berkeley DNA Sequencing Facility. For use in strain construction, the entire cassette (promoter through the drug marker) in such a plasmid was amplified by PCR and used for integrative transformation (Rothstein 1991). This strategy was used to integrate each of the septin alleles generated in this study into the corresponding endogenous chromosomal locus under control of its native promoter. Successful yeast strain construction was confirmed using isolated genomic DNA and multiple diagnostic PCR reactions to assess each affected loci after every successive round of integration. Plasmids used in this study are listed in Table 2 and were constructed using *in vivo* ligation and homologous recombination in yeast, as described above.

Culture conditions

Yeast were cultured in YP medium (1% yeast extract, 2% peptone) containing either 2% dextrose/glucose (YPD) or 2% galactose (YPGal), cultured in synthetic drop-out medium supplemented with the appropriate amino acids and containing either glucose or galactose, or cultured in synthetic drop-out medium supplemented with the appropriate amino acids containing raffinose (2%) and sucrose (0.2%) (Sherman *et al.* 1986). Growth assays were performed by spotting onto agar plates $\sim 5 \mu\text{l}$ of fivefold serial dilutions of an overnight culture of the strain(s) to be tested (first spot being roughly equivalent to $1 A_{600 \text{ nm}}$ unit of cells. For growth tests on any medium containing 5-fluoro-orotic acid (5-FOA; Oakwood Products Inc., West Columbia, SC) (final concentration of 0.5 mg/ml of medium, heated to 75° for 30 min and filter sterilized rather than autoclaved), yeast strains were always grown overnight in medium selective for the presence of the *URA3*-marked plasmid (as well as any other plasmids present). The only exception to the preceding pregrowth regimen was for strains lacking *CDC10*, in which the yeast were grown overnight at 20°–25° in YPGal medium prior to spotting to allow for more efficient loss of the *URA3*-marked covering plasmid (McMurray *et al.* 2011). For strains that did not contain any *URA3*-marked covering plasmid, cultures were grown in YPD medium at the indicated temperature; if a strain harbored any additional plasmid(s), it was grown in minimal medium supplemented with appropriate nutrients to maintain selection. For strains that required testing at high temperature (37°), strains were counterselected for all covering plasmids

(at permissive/low temperature) and growth assays were performed on appropriate medium without the need for 5-FOA selection. Growth results were scored after incubating the plates for 2–5 days, depending on the strains being tested.

Fluorescence and DIC microscopy

For imaging, exponentially growing cells were washed with water, resuspended in water, and immediately examined using an Olympus BH-2 upright fluorescence microscope (Olympus, Tokyo, Japan) equipped with a 100 $\times$ objective, illuminated with a SOLA light engine (Lumencore, Beaverton, OR) and images recorded with a CoolSNAP MYO CCD camera (Photometrics, Tuscon, AZ). Images were analyzed using Micro-Manager software (Edelstein *et al.* 2010) and ImageJ (National Institutes of Health). All images grouped together in any given figure were always scaled identically and always adjusted identically for brightness using Photoshop (Adobe). The cell perimeter was determined using either a DIC image of the same field or an overexposed fluorescence image.

Results

Shs1 is essential for viability in four different genetic backgrounds

The products of four septin genes *CDC3*, *CDC10*, *CDC11*, and *CDC12* are essential for cell growth, normal morphology, and completion of cytokinesis in *S. cerevisiae* (Hartwell 1971; Hartwell *et al.* 1974). By contrast, absence of the fifth, mitotically expressed septin gene *SHS1*, a close paralog of *CDC11* (Pan *et al.* 2007), causes rather minor and subtle phenotypes (Iwase *et al.* 2007; Egelhofer *et al.* 2008; Garcia *et al.* 2011; Buttery *et al.* 2012). We explored whether compromising the function of another septin might provide a sensitized genetic background wherein presence of *Shs1* becomes essential for viability. We found, first, that cells of the S288C lineage (Mortimer and Johnston 1986) lacking *Shs1* and endogenously expressing a derivative of *Cdc11* tagged at its C terminus with a fluorescent protein tag, mCherry (mC) (Shaner *et al.* 2004), reproducibly grew somewhat less robustly than otherwise identical cells endogenously expressing either *Cdc3*–mC or *Cdc10*–mC (Figure 1A). To examine the efficiency of incorporation of such tagged subunits into the septin structures at the bud neck, cells endogenously expressing either *Cdc10*–mC or *Cdc11*–mC (and coexpressing a second copy of WT *CDC10* or WT *CDC11*, respectively, from a *URA3*-marked *CEN* plasmid) were viewed by fluorescence microscopy, and no dramatic differences were observed in cells lacking *Shs1*, as compared to the *SHS1*⁺ control cells (Figure 1B, left). Strikingly, however, upon passage on medium containing 5-FOA (Boeke *et al.* 1984) to select for cells that had lost the covering plasmid, we found that the cells lacking *Shs1* and expressing *Cdc10*–mC exhibited normal morphology, whereas the cells lacking *Shs1* and expressing *Cdc11*–mC displayed a markedly aberrant morphology with elongated buds indicative of a defect in septin function (Figure 1B, right). Thus, even though *Cdc11*–mC does not appear

Table 1 Yeast strains used in this study

Strain	Genotype	Reference
BY4741	<i>MATa leu2Δ ura3Δ met15Δ his3Δ</i>	Brachmann <i>et al.</i> (1998)
GFY-38	BY4741; <i>shs1Δ::Kan^R</i>	This study
GFY-42	BY4741; <i>cdc10Δ::CDC10::mCherry::SpHIS5</i>	This study
GFY-6	BY4741; <i>cdc10Δ::CDC10::mCherry::SpHIS5 shs1Δ::Kan^R</i>	This study
GFY-768	BY4741; <i>cdc11Δ::CDC11::mCherry::SpHIS5</i>	This study
GFY-769	BY4741; <i>cdc11Δ::CDC11::mCherry::SpHIS5 shs1Δ::Hyg^R</i>	This study
GFY-29	BY4741; <i>cdc3Δ::CDC3::mCherry::SpHIS5</i>	This study
GFY-30	BY4741; <i>cdc3Δ::CDC3::mCherry::SpHIS5 shs1Δ::Kan^R</i>	This study
GFY-87	BY4741; <i>cdc10Δ::Kan^R shs1Δ::SHS1::eGFP::Nat^R + pJT2022</i>	This study
GFY-137	BY4741; <i>cdc10Δ::Kan^R shs1Δ::Hyg^R + pJT2022</i>	This study
GFY-293	BY4741; <i>cdc11Δ::cdc11(Δ357-415)::mCherry::SpHIS5 shs1Δ::SHS1::eGFP::Nat^R + pJT1520</i>	This study
GFY-166	BY4741; <i>cdc11Δ::cdc11(Δ357-415)::mCherry::SpHIS5 shs1Δ::Hyg^R + pJT1520</i>	This study
GFY-302 ^a	BY4741; <i>cdc12Δ::cdc12(K391N Δ392-407)::Hyg^R shs1Δ::SHS1::eGFP::Nat^R + pJT1622</i>	This study
GFY-139 ^a	BY4741; <i>cdc12Δ::cdc12(K391N Δ392-407)::Hyg^R shs1Δ::Kan^R + pJT1622</i>	This study
YFR-387 ^b	BY4742; <i>hof1Δ::Kan^R + pJT4836</i>	This study
GFY-934	BY4742; <i>hof1Δ::Kan^R shs1Δ::Hyg^R + pJT4836</i>	This study
GFY-95 ^c	BY4741; <i>cdc10Δ::Kan^R shs1Δ::shs1(T6A S447A)::eGFP::Nat^R + pJT2022</i>	This study
GFY-96 ^c	BY4741; <i>cdc10Δ::Kan^R shs1Δ::shs1(T6A T386A S416A S441A S447A)::eGFP::Nat^R + pJT2022</i>	This study
GFY-97 ^c	BY4741; <i>cdc10Δ::Kan^R shs1Δ::shs1(T386A S416A S441A S447A S460A T462A S519A S520A S521A S522A S525A S545A)::eGFP::Nat^R + pJT2022</i>	This study
GFY-191 ^c	BY4741; <i>cdc10Δ::Kan^R shs1Δ::shs1(S350A T351A T386A S416A S441A S447A T454A S460A T462A S519A S520A S521A S522A S525A S529A S530A T539A T541A S545A)::eGFP::Nat^R + pJT2022</i>	This study
GFY-189 ^d	BY4741; <i>cdc10Δ::Kan^R shs1Δ::shs1(S259A)::eGFP::Nat^R + pJT2022</i>	This study
GFY-88 ^e	BY4741; <i>cdc10Δ::Kan^R shs1Δ::shs1(T6D)::eGFP::Nat^R + pJT2022</i>	This study
GFY-192 ^e	BY4741; <i>cdc10Δ::Kan^R shs1Δ::shs1(T6D T386D S416D S441D S447D)::eGFP::Nat^R + pJT2022</i>	This study
GFY-90 ^e	BY4741; <i>cdc10Δ::Kan^R shs1Δ::shs1(T386D S416D S441D S447D S460D T462D S519D S520D S521D S522D S525D S545D)::eGFP::Nat^R + pJT2022</i>	This study
GFY-91 ^e	BY4741; <i>cdc10Δ::Kan^R shs1Δ::shs1(S259D)::eGFP::Nat^R + pJT2022</i>	This study
GFY-310 ^e	BY4741; <i>cdc10Δ::Kan^R shs1Δ::shs1(S221D)::eGFP::Nat^R + pJT2022</i>	This study
GFY-188	BY4741; <i>cdc10Δ::Kan^R shs1Δ::shs1(S221A)::eGFP::Nat^R + pJT2022</i>	This study
GFY-160	BY4741; <i>cdc11Δ::CDC11::mCherry::SpHIS5^R shs1Δ::SHS1::eGFP::Nat^R + pJT1520</i>	This study
GFY-147	BY4741; <i>cdc11Δ::Kan^R shs1Δ::SHS1::eGFP::Nat^R + pJT1520</i>	This study
GFY-164	BY4741; <i>cdc11Δ::CDC11::mCherry::SpHIS5^R shs1Δ::Hyg^R + pJT1520</i>	This study
GFY-163 ^f	BY4741; <i>cdc11Δ::Kan^R shs1Δ::Hyg^R + pJT1520</i>	This study
GFY-437 ^g	BY4741; <i>cdc11Δ::Nat^R shs1Δ::Hyg^R cdc12Δ::cdc12(W267A)::Kan^R + pJT1520/pJT1622</i>	This study
GFY-369	BY4741; <i>cdc10Δ::Kan^R shs1Δ::shs1(G30D)::eGFP::Nat^R + pJT2022</i>	This study
GFY-249	BY4741; <i>cdc10Δ::Kan^R shs1Δ::shs1(R13A R14A K15A K16A K19A R20A)::eGFP::Nat^R + pJT2022</i>	This study
GFY-93	BY4741; <i>cdc10Δ::Kan^R shs1Δ::shs1(Δ2-18)::eGFP::Nat^R + pJT2022</i>	This study
GFY-58	BY4741; <i>cdc11Δ::CDC11::mCherry::SpHIS5 + pJT1520</i>	This study
GFY-121	BY4741; <i>cdc11Δ::cdc11(G29D)::mCherry::Kan^R + pJT1520</i>	This study
GFY-308	BY4741; <i>cdc11Δ::cdc11(G29D)::mCherry::Kan^R shs1Δ::Hyg^R + pJT1520</i>	This study
GFY-375	BY4741; <i>cdc11Δ::cdc11(G29D)::mCherry::Kan^R shs1Δ::shs1(G30D)::eGFP::Nat^R + pJT1520</i>	This study
GFY-397	BY4741; <i>cdc11Δ::Kan^R shs1Δ::shs1(G30D)::eGFP::Nat^R + pJT1520</i>	This study
GFY-843 ^h	BY4741; <i>cdc11Δ::Kan^R shs1Δ::shs1(G30D)::eGFP::Nat^R cdc12Δ::cdc12(W267A)::SpHIS5 + pJT1520/pJT1622</i>	This study
GFY-246 ⁱ	BY4741; <i>cdc11Δ::cdc11(R12A K13A R14A K15A K18A R19A)::mCherry::SpHIS5 + pJT1520</i>	This study
GFY-247	BY4741; <i>cdc11Δ::cdc11(R12A K13A R14A K15A K18A R19A)::mCherry::SpHIS5 shs1Δ::Hyg^R + pJT1520</i>	This study
GFY-307	BY4741; <i>cdc11Δ::cdc11(R12A K13A R14A K15A K18A R19A)::mCherry::SpHIS5 shs1Δ::shs1(R13A R14A K15A K16A K19A R20A)::eGFP::Nat^R + pJT1520</i>	This study
GFY-253	BY4741; <i>cdc11Δ::Kan^R shs1Δ::shs1(R13A R14A K15A K16A K19A R20A)::eGFP::Nat^R + pJT1520</i>	This study
GFY-123	BY4741; <i>cdc11Δ::cdc11(Δ2-18)::mCherry::SpHIS5^R + pJT1520</i>	This study
GFY-165	BY4741; <i>cdc11Δ::cdc11(Δ2-18)::mCherry::SpHIS5^R shs1Δ::Hyg^R + pJT1520</i>	This study
GFY-161	BY4741; <i>cdc11Δ::cdc11(Δ2-18)::mCherry::SpHIS5^R shs1Δ::shs1(2-18Δ)::eGFP::Nat^R + pJT1520</i>	This study
GFY-148	BY4741; <i>cdc11Δ::Kan^R shs1Δ::shs1(Δ2-18)::eGFP::Nat^R + pJT1520</i>	This study
GFY-94	BY4741; <i>cdc10Δ::Kan^R shs1Δ::shs1(Δ349-551)::eGFP::Nat^R + pJT2022</i>	This study
GFY-98	BY4741; <i>cdc10Δ::Kan^R shs1Δ::shs1(Δ465-496)::eGFP::Nat^R + pJT2022</i>	This study
GFY-178 ^j	BY4741; <i>cdc10Δ::Kan^R shs1Δ::shs1(Y465A R468A I472A R475A L479A L482A S486A E489A R493A E496A)::eGFP::Nat^R + pJT2022</i>	This study
GFY-122	BY4741; <i>cdc11Δ::cdc11(Δ357-415)::mCherry::SpHIS5 + pJT1520</i>	This study

(continued)

Table 1, continued

Strain	Genotype	Reference
GFY-162	BY4741; <i>cdc11Δ::cdc11(Δ357-415)::mCherry::SpHIS5 shs1Δ::shs1(Δ349-551)::eGFP::Nat^R + pJT1520</i>	This study
GFY-149	BY4741; <i>cdc11Δ::Kan^R shs1Δ::shs1(Δ349-551)::eGFP::Nat^R + pJT1520</i>	This study
GFY-378 ⁱ	BY4741; <i>cdc11Δ::cdc11(E377A V380A L384A K387A L391A R394A L398A I401A L405A E408A K412A E415A)::mCherry::SpHIS5 + pJT1520</i>	This study
GFY-383 ^j	BY4741; <i>cdc11Δ::cdc11(E377A V380A L384A K387A L391A R394A L398A I401A L405A E408A K412A E415A)::mCherry::SpHIS5 shs1Δ::Hyg^R + pJT1520</i>	This study
GFY-475 ^j	BY4741; <i>cdc11Δ::cdc11(E377A V380A L384A K387A L391A R394A L398A I401A L405A E408A K412A E415A)::mCherry::SpHIS5 shs1Δ::shs1(Y465A R468A I472A R475A L479A L482A S486A E489A R493A E496A)::eGFP::Nat^R + pJT1520</i>	This study
GFY-233	BY4741; <i>cdc10Δ::Kan^R shs1Δ::shs1(Δ342-352)::eGFP::Nat^R + pJT2022</i>	This study
GFY-234	BY4741; <i>cdc10Δ::Kan^R shs1Δ::shs1(Δ353-379)::eGFP::Nat^R + pJT2022</i>	This study
GFY-235	BY4741; <i>cdc10Δ::Kan^R shs1Δ::shs1(Δ380-404)::eGFP::Nat^R + pJT2022</i>	This study
GFY-236	BY4741; <i>cdc10Δ::Kan^R shs1Δ::shs1(Δ405-429)::eGFP::Nat^R + pJT2022</i>	This study
GFY-237	BY4741; <i>cdc10Δ::Kan^R shs1Δ::shs1(Δ430-450)::eGFP::Nat^R + pJT2022</i>	This study
GFY-519	BY4741; <i>cdc10Δ::Kan^R shs1Δ::shs1(Δ342-386)::eGFP::Nat^R + pJT2022</i>	This study
GFY-503	BY4741; <i>cdc10Δ::Kan^R shs1Δ::shs1(Δ387-436)::eGFP::Nat^R + pJT2022</i>	This study
GFY-514	BY4741; <i>cdc10Δ::Kan^R shs1Δ::shs1(Δ342-436)::eGFP::Nat^R + pJT2022</i>	This study
GFY-381	BY4741; <i>cdc11Δ::cdc11(Δ301-313)::mCherry::SpHIS5 shs1Δ::Hyg^R + pJT1520</i>	This study
GFY-398	BY4741; <i>cdc11Δ::cdc11(Δ314-329)::mCherry::SpHIS5 shs1Δ::Hyg^R + pJT1520</i>	This study
GFY-399	BY4741; <i>cdc11Δ::cdc11(Δ330-343)::mCherry::SpHIS5 shs1Δ::Hyg^R + pJT1520</i>	This study
GFY-382	BY4741; <i>cdc11Δ::cdc11(Δ344-357)::mCherry::SpHIS5 shs1Δ::Hyg^R + pJT1520</i>	This study
GFY-513	BY4741; <i>cdc11Δ::cdc11(Δ301-329)::mCherry::SpHIS5 shs1Δ::Hyg^R + pJT1520</i>	This study
GFY-524	BY4741; <i>cdc11Δ::cdc11(Δ330-357)::mCherry::SpHIS5 shs1Δ::Hyg^R + pJT1520</i>	This study
GFY-512	BY4741; <i>cdc11Δ::cdc11(Δ301-357)::mCherry::SpHIS5 shs1Δ::Hyg^R + pJT1520</i>	This study
GFY-676	BY4741; <i>cdc11Δ::cdc11(Δ301-357)::mCherry::SpHIS5 shs1Δ::shs1(342-436Δ)::eGFP::Nat^R + pJT1520</i>	This study
GFY-126	BY4741; <i>cdc3Δ::CDC3::mCherry::SpHIS5 + pJT2059</i>	This study
YMBVB33	BY4741; <i>cdc3Δ::Kan^R + pJT2059</i>	McMurray et al. (2011)
GFY-501	BY4741; <i>cdc3Δ::cdc3(Δ440-520)::mCherry::SpHIS5 + pJT2059</i>	This study
GFY-764 ⁱ	BY4741; <i>cdc3Δ::cdc3(E467A F471A H474A K478A L481A L485A L488A K492A L495A I499A V506A)::mCherry::SpHIS5 + pJT2059</i>	This study
GFY-494	BY4741; <i>cdc3Δ::cdc3(Δ412-423)::mCherry::SpHIS5 + pJT2059</i>	This study
GFY-493	BY4741; <i>cdc3Δ::cdc3(Δ424-435)::mCherry::SpHIS5 + pJT2059</i>	This study
GFY-507	BY4741; <i>cdc3Δ::cdc3(Δ436-450)::mCherry::SpHIS5 + pJT2059</i>	This study
GFY-511	BY4741; <i>cdc3Δ::cdc3(Δ412-450)::mCherry::SpHIS5 + pJT2059</i>	This study
GFY-251	BY4741; <i>cdc12Δ::CDC12::mCherry::Kan^R + pJT1622</i>	This study
YMBVB61	BY4741; <i>cdc12Δ::Kan^R + pJT1622</i>	McMurray et al. (2011)
GFY-515	BY4741; <i>cdc12Δ::cdc12(Δ339-407)::mCherry::Kan^R + pJT1622</i>	This study
GFY-535 ^j	BY4741; <i>cdc12Δ::cdc12(E368A I371A R375A L478A L482A, I485A V489A L492A V496A L499A K403A L406A)::mCherry::Kan^R + pJT1622</i>	This study
GFY-516	BY4741; <i>cdc12Δ::cdc12(Δ315-326)::mCherry::Kan^R + pJT1622</i>	This study
GFY-498	BY4741; <i>cdc12Δ::cdc12(Δ327-338)::mCherry::Kan^R + pJT1622</i>	This study
GFY-510	BY4741; <i>cdc12Δ::cdc12(Δ339-353)::mCherry::Kan^R + pJT1622</i>	This study
GFY-525	BY4741; <i>cdc12Δ::cdc12(Δ315-353)::mCherry::Kan^R + pJT1622</i>	This study
GFY-179	BY4741; <i>cdc10Δ::Kan^R shs1Δ::shs1(Y465A)::eGFP::Nat^R + pJT2022</i>	This study
GFY-180	BY4741; <i>cdc10Δ::Kan^R shs1Δ::shs1(R468A)::eGFP::Nat^R + pJT2022</i>	This study
GFY-181	BY4741; <i>cdc10Δ::Kan^R shs1Δ::shs1(I472A)::eGFP::Nat^R + pJT2022</i>	This study
GFY-362	BY4741; <i>cdc10Δ::Kan^R shs1Δ::shs1(R475A)::eGFP::Nat^R + pJT2022</i>	This study
GFY-368	BY4741; <i>cdc10Δ::Kan^R shs1Δ::shs1(L479A)::eGFP::Nat^R + pJT2022</i>	This study
GFY-366	BY4741; <i>cdc10Δ::Kan^R shs1Δ::shs1(L482A)::eGFP::Nat^R + pJT2022</i>	This study
GFY-367	BY4741; <i>cdc10Δ::Kan^R shs1Δ::shs1(S486A)::eGFP::Nat^R + pJT2022</i>	This study
GFY-363	BY4741; <i>cdc10Δ::Kan^R shs1Δ::shs1(E489A)::eGFP::Nat^R + pJT2022</i>	This study
GFY-391	BY4741; <i>cdc10Δ::Kan^R shs1Δ::shs1(R493A)::eGFP::Nat^R + pJT2022</i>	This study
GFY-370	BY4741; <i>cdc10Δ::Kan^R shs1Δ::shs1(E496A)::eGFP::Nat^R + pJT2022</i>	This study
GFY-544 ^k	BY4741; <i>cdc11Δ::cdc11(1-308)::shs1(349-551)::mCherry::Kan^R shs1Δ::Hyg^R + pJT1520</i>	This study
GFY-540 ^k	BY4741; <i>cdc11Δ::cdc11(Δ357-415)::mCherry::SpHIS5 shs1Δ::shs1(1-348)::cdc11(309-415)::eGFP::Nat^R + pJT1520</i>	This study

(continued)

Table 1, continued

Strain	Genotype	Reference
GFY-542 ^k	BY4741; <i>cdc11Δ::cdc11(1-308)::shs1(349-551)::mCherry::Kan^R shs1Δ::shs1(Δ349-551)::eGFP::Nat^R + pJT1520</i>	This study
GFY-692 ^k	BY4741; <i>cdc11Δ::cdc11(1-308)::shs1(349-551)::mCherry::Kan^R shs1Δ::shs1(1-348)::cdc11(309-415)::eGFP::Nat^R + pJT1520</i>	This study
GFY-548 ^k	BY4741; <i>cdc10Δ::Kan^R shs1Δ::shs1(1-348)::cdc11(309-415)::eGFP::Nat^R + pJT2022</i>	This study

^a It was reported that the *cdc12-6* allele is a frameshift mutation arising from insertion of a single A into a tract of seven adenines (nucleotides 1167–1173) in the *CDC12* ORF (Longtine *et al.* 2000); however, the original sequencing data (B. Haarer, M. Longtine, and J. Pringle, personal communication) show that *cdc12-6* is a frameshift arising from deletion of a single A from this same adenine tract, resulting in a substitution mutation (K391N) immediately followed by a stop codon (TAG), thereby causing truncation of the remainder of the protein (Δ392–407). As documented elsewhere, loss of the C-terminal residues (rather than the K391N substitution) is responsible for the ts phenotype conferred by *cdc12-6* (Johnson *et al.* 2015). Our *cdc12-6* construct arises from removal of a single A (nucleotide 1173) from the *CDC12* ORF, producing the frameshift that results in substitution mutation K391N immediately followed by the nonsense codon that truncates the remainder of the protein and has the *ADH1* transcriptional terminator sequence and a *Hyg^R* drug resistant marker inserted immediately after nucleotide 1224.

^b JTY5682 (*hof1Δ::Kan^R/HOF1* heterozygous diploid) was transformed with pJT4836 (pRS316–*HOF1*), sporulated, and the resulting tetrads dissected to isolate strain YFR-387.

^c Each *shs1* phosphonull mutant was amplified, respectively, from genomic DNA of yeast strains DK966, DK912, DK1033, and DK985 (Egelhofer *et al.* 2008).

^d To introduce a mutation into an ORF, e.g., *Shs1*(S221A) encoded by GFY-188, a modified Quikchange PCR protocol (Zheng *et al.* 2004) was carried out using as the template the gene of interest inserted into a TOPO-II vector (Life Technologies, Inc.). To introduce multiple mutations, e.g., *Shs1*(R13A R14A K15A K16A K19A R20A) encoded by GFY-249, successive rounds of Quikchange were used.

^e Each *shs1* phosphomimetic allele was PCR amplified from previously constructed expression vectors harboring these mutations (Garcia *et al.* 2011).

^f To create GFY-163, the *cdc11Δ::SkHIS3* locus from JT3253 was cloned into a TOPO-II vector with 300 bp of its flanking DNA both 5' and 3'; the knock-out cassette is in the reverse orientation to that of the *CDC11* ORF. Following subcloning into pRS315, the *SkHIS3* cassette was excised and the resulting gapped plasmid cotransformed with a PCR product containing the *Kan^R* cassette flanked by sequences homologous to those flanking the *CDC11* ORF. *In vivo* ligation in yeast generated *cdc11Δ::Kan^R* in which the inserted cassette has the same orientation as the *CDC11* ORF. The *cdc11Δ::Kan^R* cassette and its flanking DNA were amplified by PCR, treated with *DpnI* enzyme, and transformed into WT yeast (BY4741) that also contained a *URA3*-marked plasmid (pJT1520) expressing WT untagged *CDC11*. Proper substitution of the endogenous *CDC11* locus with the deletion cassette in *Kan^R* clones was confirmed in two ways. First, clonal isolates were tested for inviability at 30° upon streaking twice on 5-FOA medium (to select against the covering plasmid). Second, genomic DNA was prepared from the apparent *cdc11Δ::Kan^R* isolates and analyzed by PCR using primers internal to the *Kan^R* gene and primers upstream of the 5'-flanking region of the *cdc11Δ::Kan^R* construct. Finally, a confirmed *cdc11Δ::Kan^R* isolate was transformed with DNA containing the *shs1Δ::Hyg^R* knock-out cassette and 500 bp of its flanking regions both 5' and 3', which was generated by PCR amplification of DNA from strain JT5324, yielding strain GFY-163.

^g To construct strain GFY-437, a *MATa cdc11Δ::Nat^R shs1Δ::Hyg^R* double mutant (harboring a *URA3*-marked *CEN* plasmid expressing WT *CDC11*) was mated against a *MATα cdc12Δ::cdc12(W267A)::Kan^R* strain (harboring a *URA3*-marked *CEN* plasmid expressing WT *CDC12*), the latter generated from sporulation and dissection of JT3619 and a subsequent drug marker swap). After mating, *Nat^R Hyg^R Kan^R* diploids were selected on medium containing Geneticin (Gibco/Life Technologies, Grand Island, NY), ClonNat (Werner BioAgents, Jena, Germany), Hygromycin (EMD Millipore Biosciences, Billerica, MA), sporulated, the resulting tetrads dissected, and the desired *cdc11Δ::Nat^R shs1Δ::Hyg^R cdc12Δ::cdc12(W267A)::Kan^R* haploid spores selected using the same three antibiotics. The final spore clone chosen must contain at least the covering *CDC11* plasmid (but may also contain the *CDC12* plasmid).

^h To create strain GFY-843, the marker in GFY-843 was swapped (*cdc11Δ::Nat^R* to *cdc11Δ::Kan^R*) before integrating *shs1(G30D)::eGFP::Nat^R* at the *SHS1* locus.

ⁱ For GFY-246, the *cdc11* gene was amplified from YCp111/*cdc11AS* (McMurray *et al.* 2011).

^j The following strategy was used to construct strains expressing derivatives of Cdc3, Cdc11, Cdc12, and Shs1 in which 10–12 residues corresponding to the predicted coiled-coil-forming segment in the CTE were substituted with Ala ("CC-A" mutants). A 455-bp fragment of each septin gene including the Ala substitutions was synthesized (GenScript, Piscataway, NJ). The fragment containing the mutations was PCR amplified with primers that had upstream homology to the remaining gene sequence and downstream homology to link the fragment to either eGFP or mCherry, and then *in vivo* ligation with the remainder of each septin gene containing overlap to the synthetic mutant fragment was performed to reassemble each of the septin genes.

^k For clarity, the nomenclature for the chimeras between Cdc11 and Shs1 indicates the residues present in each of the hybrid proteins (rather than any residues that may have been deleted).

functionally deficient when *Shs1* is present (Supporting Information, Figure S1A), our results revealed that *Cdc11* is somewhat impaired when mC is fused to its CTE because overall septin function became sensitive to the absence of *Shs1*.

To explore this possibility further, we perturbed *Cdc11* even more by fusing mC to a *Cdc11* derivative in which its CTE had been deleted. Even though the CTE of *Cdc11* is not required for cell viability when *Shs1* is present (Versele *et al.* 2004), or for filament formation *in vitro* (Bertin *et al.* 2008), we found, in agreement with our expectation, that cells expressing *Cdc11*(ΔCTE)–mC as the sole source of this septin were unable to propagate at all when *Shs1* was absent, but grew essentially normally when *Shs1* was present (Figure 1C, top). In a similar fashion, we systematically explored other known circumstances that partially cripple the function of septins and were able to find three other conditions under which expression of *SHS1* becomes essential for cell viability:

i. On galactose medium and at 25°, cells lacking *Cdc10* are able to survive (McMurray *et al.* 2011), but, we found that, under the same conditions, such cells were not viable if

they also lacked *Shs1* (Figure 1C, top middle), confirming an observation made by Iwase *et al.* (2007). Either *Shs1* or *Shs1*–eGFP was able to restore viability (Figure S1B).

ii. Likewise, a frameshift mutation in *CDC12* that generates a temperature-sensitive substitution-truncation allele [*cdc12-6*; *Cdc12*(K391N E392Stop)] (Johnson *et al.* 2015) grows well at 25° (Dobbelaere and Barral 2004; Gladfelter *et al.* 2005; Nagaraj *et al.* 2008; Weems *et al.* 2014), whereas we found that such cells were inviable at this otherwise permissive temperature if they also lacked *Shs1* (Figure 1C, bottom middle).

iii. Finally, in agreement with an observation made by Meitinger *et al.* (2013), we found that cells lacking *Hof1*, a bud neck-localized, F-BAR- and SH3 domain-containing protein that regulates actomyosin ring dynamics (Nishihama *et al.* 2009; Meitinger *et al.* 2011), are inviable if *Shs1* is absent (Figure 1C, bottom).

Collectively, these results indicated that, far from being dispensable, *Shs1* does contribute significantly to optimal septin function *in vivo* and provided the means to delineate

Table 2 Plasmids used in this study

Plasmid	Description	Reference
pSB1/JT1520	pRS316 <i>URA3 CDC11</i>	Versele <i>et al.</i> (2004)
pMVB57/JT2022	YCplac33 <i>URA3 CDC10</i>	McMurray <i>et al.</i> (2011)
pMVB39/JT1622	YCplac33 <i>URA3 CDC12</i>	Versele <i>et al.</i> (2004)
pMVB100/JT2059	YCplac33 <i>URA3 CDC3</i>	Versele <i>et al.</i> (2004)
pJT4836	pRS316 <i>prHOF1::HOF1</i>	This study
pGF-IVL1	pRS315 <i>prCDC11::CDC11::mCherry::SpHIS5</i>	This study
pRS315	<i>CEN, LEU2</i>	Sikorski and Hieter (1989)
pGF-preIVL6	pRS315 <i>prCDC10::CDC10::mCherry::SpHIS5</i>	This study
pGF-IVL370	pRS315 <i>prCDC10::CDC10::shs1(349-551)::eGFP::Nat^R</i>	This study
pGF-IVL371	pRS315 <i>prCDC10::CDC10::cdc11(309-415)::GFP::Nat^R</i>	This study
pGF-preIVL59	pRS315 <i>prSHS1::SHS1::eGFP::Nat^R</i>	This study
pGF-preIVL60	pRS315 <i>shs1Δ::shs1(T6D)::eGFP::Nat^R</i>	This study
pGF-preIVL21	pRS313 <i>prSHS1::shs1(T6D T386D S416D S441D S447D)::eGFP::Nat^R</i>	This study
pGF-preIVL22	pRS313 <i>prSHS1::shs1(T386D S416D S441D S447D S460D T462D S519D S520D S521D S522D S525D S545D)::eGFP::Nat^R</i>	This study
pGF-preIVL23	pRS313 <i>prSHS1::shs1(S259D)::eGFP::Nat^R</i>	This study
pGF-preIVL62	pRS315 <i>prSHS1::shs1(T6A S447A)::eGFP::Nat^R</i>	This study
pGF-preIVL63	pRS315 <i>prSHS1::shs1(T6A T386A S416A S441A S447A)::eGFP::Nat^R</i>	This study
pGF-preIVL64	pRS315 <i>prSHS1::shs1(T386A S416A S441A S447A S460A T462A S519A S520A S521A S522A S525A S545A)::eGFP::Nat^R</i>	This study
pGF-IVL25	pRS315 <i>prSHS1::shs1(S259A)::eGFP::Nat^R</i>	This study

specific domains and/or residues within *Shs1* that contribute to its physiological role, which we could corroborate in several independent genetic backgrounds. (See Table S1.)

For simplicity and clarity, we are documenting our results by showing primarily data obtained using the *Shs1*-dependent growth of *cdc10Δ* cells on Gal medium at 25°. However, as we also show in multiple instances, the same phenotypes were manifest in one or more of the other sensitized *Shs1*-dependent genetic backgrounds just described, thereby verifying all of our primary conclusions in more than one cellular context. In all cases, our mutants were integrated and expressed from the corresponding endogenous chromosomal locus.

Phosphorylation of its CTE is not required for *Shs1* function

The first question we chose to address in this regard pertained to the numerous sites of cell-cycle-dependent phosphorylation in *Shs1* that have been previously reported (Mortensen *et al.* 2002; Dobbelaere *et al.* 2003; Egelhofer *et al.* 2008), but whose functional significance, if any, has not yet been discerned. Others have generated nonphosphorylatable (Ser- or Thr-to-Ala) mutations and/or phosphomimetic (Ser- or Thr-to-Asp) alleles at positions determined to be phosphorylated either *in vitro* or *in vivo* (Dobbelaere *et al.* 2003; Egelhofer *et al.* 2008; Garcia *et al.* 2011). Hence, one set of *Shs1* mutations we tested were nonphosphorylatable alleles (Egelhofer *et al.* 2008): P1(A), a single mutation (T6A) near the *Shs1* N terminus; P2(A), T6A plus four sites in the CTE (T386A, S416A, S441A and S447A); P3(A), P2 plus eight more sites in the CTE (S460A, T462A, S519A, S520A, S521A, S522A, S525A and S545A); P4(A) 19 sites exclusively in the CTE (S350A, T351A, T386A, S416A, S441A, S447A, T454A, S460A, T462A, S519A, S520A, S521A, S522A, S525A, S529A, S530A,

T539A, T541A and S545A); and, a single mutation (S259A) in the globular GTP-binding domain located at neither its G or NC interfaces, based on homology modeling (Bertin *et al.* 2010). Adjacent monomers in linear septin complexes interact by two alternating contact modes, a “G interface” (involving residues in and around the GTP-binding pockets), and an “NC interface” (involving residues in and around the N- and C-terminal segments of the GTP-binding domains). *Cdc12* interacts with *Cdc3* by an NC interface (Bertin *et al.* 2008) and must therefore interact with *Shs1* via a G interface. Another set of *Shs1* mutations we tested were corresponding phosphomimetic alleles: P1(D), P2(D), P3(D), and, S259D (Garcia *et al.* 2011). A third pair of alleles we tested were S221A and S221D at a site phosphorylated by *Rad53* in response to DNA damage (Enserink *et al.* 2006; Smolka *et al.* 2006; Smolka *et al.* 2007; Albuquerque *et al.* 2008).

Using three different genetic backgrounds in which growth is dependent on the presence of functional *Shs1*, we found that, like WT *Shs1*, all of the nonphosphorylatable alleles and all of the phosphomimetic alleles at putative CDK and *Gen4* sites were able to support growth (Figure 1, D, E, and F). The sole exception was the P3(D), which appeared to be severely crippled for function, but only in the *cdc10Δ* background (Figure 1D). This result suggests that, in the absence of *Cdc10* uniquely, the cumulative effect on the electrostatic and presumably protein-binding properties of the CTE of *Shs1* (due to introduction of 13 nonnative negatively charged residues) is deleterious to overall septin function. For this reason, and because it would contain 19 new negatively charged residues, we did not construct and test a P4(D) allele. It is important to note, however, that the lack of complementation by P3(D) was not due to lack of stable protein because it (and all of the other alleles examined here) was expressed in *cdc10Δ* cells at a level equivalent to WT *Shs1*

Figure 1 Sensitized genetic backgrounds permit analysis of Shs1 function. (A) Overnight cultures of the indicated genotypes (strains BY4741, GFY-38, GFY-42, GFY-6, GFY-768, GFY-769, GFY-29, and GFY-30) were spotted in five-fold serial dilutions onto rich medium plates and grown at 30° for 2 days. (B) Exponentially growing cultures of the indicated genotype, in which the mCherry-tagged septin was expressed from its endogenous locus and the cells also expressed a plasmid-borne copy of the corresponding WT septin, were examined first by fluorescence microscopy (left) and then, after selection for loss of the covering plasmid on 5-FOA medium, by DIC microscopy (right). Dotted white line, cell periphery; arrowheads, cells with abnormal morphology. (C) Otherwise isogenic strain pairs of the indicated genotype containing or lacking *SHS1*, respectively, and harboring a *URA3*-marked *CEN* plasmid expressing the indicated WT gene—GFY-293 and GFY-166 (top), GFY-87 and GFY-137 (second row), GFY-302 and GFY-139 (third row), and YFR-387 and GFY-934 (bottom)—were grown overnight in SD–Ura at 30°, spotted as in A onto the appropriate medium with glucose (D, dextrose) as the carbon source in the absence (left) and presence (right) of 5-FOA to select against the covering *URA3*-marked plasmid, and incubated at 30° for 3 days, except the *cdc10Δ* cells, which were grown in YPGal media at 25° before spotting and incubated at room temperature for 5 days, and the *cdc12-6* cells, which were propagated and incubated at a permissive temperature (25°). In GFY-293, GFY-87, and GFY-302, the endogenous *SHS1* allele was C terminally tagged with eGFP. The *cdc11Δ* (CTE) allele in GFY-293 and GFY-166 was tagged with mCherry (mC). (D) The indicated *shs1* alleles were expressed in *cdc10Δ* cells (GFY-87, GFY-137, GFY-95, GFY-96, GFY-97, GFY-191, GFY-189, GFY-88, GFY-192, GFY-90, GFY-91, GFY-310, and GFY-188) and tested for ability to support growth in the presence (left) and absence (right) of the covering *CDC10*-expressing plasmid, as in C. (E) A *cdc12-6 shs1Δ* strain (GFY-139) was transformed with plasmids expressing the indicated *shs1* alleles (pRS315, pGF-preVL59, pGF-pre60, pGF-preVL21, pGF-preVL22, pGF-preVL23, pGF-preVL62, pGF-preVL63, pGF-preVL64, and pGF-IVL25), grown overnight in SD–Ura–Leu at 30°, and tested for ability to support growth at 25° in the presence (left) and absence (right) of the covering *CDC12*-expressing plasmid, as in C. Transformants expressing the P2(D), P3(D), and S259D *shs1* alleles were grown overnight in SD–Ura–His at 30° and then tested for ability to support growth at 25° on SD–Ura–His (left) and SD–His + 5-FOA (right). (F) A *hof1Δ shs1Δ* strain (GFY-934) was transformed with plasmids as in E, grown overnight, and tested for ability to support growth at 30° in the presence (left) and absence (right) of the covering *HOF1*-expressing plasmid, as in C. In E and F, plates were scored after incubation for 3 days.

and localized at the bud neck (data not shown). Thus, phosphorylation at none of the CDK (*Cdc28* and *Pho85*) or *Gin4* sites previously mapped *in vivo* and *in vitro* is required for *Shs1* function in the cell, contrary to prior claims for some regulatory role for these modifications (Mortensen *et al.* 2002; Dobbelaere *et al.* 2003; Egelhofer *et al.* 2008). Similarly, even the P4(A) mutant was able to support growth in *hof1Δ* cells (Figure S1E), contrary to a suggestion that lethality of *hof1Δ shs1Δ* cells is somehow connected to a role for this septin subunit as a substrate for *Gin4* (Meitinger *et al.* 2013).

In marked contrast, one allele that mimics a permanently phosphorylated state at just a single residue, S221D, another site located on the surface of the GTP-binding domain away

from either the G or NC interface, was unable to support growth in any of the four *Shs1*-dependent strain backgrounds, whereas *Shs1*(S221A) did (Figure 1D and Figure S1C). Again, this lack of complementation was not due to lack of expression or proper folding because *Shs1*(S221D) was produced at a level equivalent to WT *Shs1* and incorporated at the bud neck (Figure S1D). Moreover, *Shs1*(S221D) appeared to be competent to associate with *Cdc12* as assessed by the independent criterion of blocking the growth of a *cdc11Δ shs1Δ* double mutant as effectively as WT *Shs1* or *Shs1*(S221A) (Figure S1C, bottom). Taken together, these findings are consistent with the possibility that cell cycle delay and morphological elongation elicited by DNA damage may arise, at least in part, from transient Rad53-mediated

phosphorylation of S221, thereby altering some *Shs1*-dependent aspect of septin architecture at the bud neck with an ensuing temporary disruption to septin function (Enserink *et al.* 2006; Smolka *et al.* 2006).

Cdc11 and Shs1 occupy the terminal position within a septin hetero-octamer in vivo

Among the budding yeast septins, *Cdc11* and *Shs1* are the most closely related subunits, most likely arising from a relatively recent gene duplication within the fungal clade (Pan *et al.* 2007). Biochemical analysis of purified recombinant *S. cerevisiae* septin complexes demonstrated that *Shs1* is able to occupy only the terminal position in septin hetero-octamers (Garcia *et al.* 2011), the same position occupied by *Cdc11* (Bertin *et al.* 2008). Thus, *in vitro*, both *Cdc11* and *Shs1* are capable of binding to *Cdc12* via a G interface interaction, thereby capping each end of the linear rod. Given their potential to serve overlapping and/or competing roles, we sought to further examine *Cdc11* and *Shs1* function *in vivo*. First, however, we wanted to ensure that the integrated alleles and new strains uniquely generated for this study recapitulated our prior conclusions about the interplay between *Cdc11* and *Shs1* obtained by using other strains and constructs (McMurray *et al.* 2011). Here, otherwise isogenic cells of different genetic constitution where any allele of interest is expressed from its endogenous chromosomal locus were covered by a *URA3*-marked plasmid expressing WT *CDC11*, which was selected against on 5-FOA medium to reveal any growth phenotype. As we have shown before (McMurray *et al.* 2011), cells lacking *CDC11* are inviable (Figure 2A, compare top line to second lane), whereas loss of *SHS1* showed very little growth defect under standard conditions (30°), even in cells where the sole source of *Cdc11* is *Cdc11-mC* (Figure 2A, compare top line to third line), although the cells become somewhat more elongated (Figure 2B, compare left top to right top). Strikingly, as we have also shown before (McMurray *et al.* 2011), removal of *SHS1* restores viability to cells lacking *Cdc11* (Figure 2, compare top line to fourth line down), even though their morphology is grossly abnormal (Figure 2B). We attributed the viability of *cdc11Δ shs1Δ* cells to the capacity of *Cdc12-Cdc3-Cdc10-Cdc10-Cdc3-Cdc12* heterohexamers to form filaments, albeit of nonnative structure, via homotypic *Cdc12-Cdc12* G interface contact (McMurray *et al.* 2011); correspondingly, as observed previously (McMurray *et al.* 2011), mutation of a residue in *Cdc12* critical for such interaction (W267A) kills *cdc11Δ shs1Δ* cells (Figure 2A, compare fifth line to fourth line), whereas it has no effect on *CDC11+* cells (Figure 2A, compare left to right side of fifth line).

By the same reasoning, the explanation for the inviability of *cdc11Δ SHS1* cells is that the presence of *Shs1* caps the ends of *Cdc12-Cdc3-Cdc10-Cdc10-Cdc3-Cdc12* heterohexamers via a G interface with *Cdc12* and blocks the formation of any filaments because *Shs1-Cdc12-Cdc3-Cdc10-Cdc10-Cdc3-Cdc12-Shs1* hetero-octamers are incapable of filament formation, at least *in vitro* (Garcia *et al.* 2011). If this explanation is correct, then *cdc11Δ* cells expressing any *Shs1* mu-

tant unable to form a G interface with *Cdc12* should also be viable, a premise not previously directly tested. Unlike elimination of the hydrophobic contact, *Cdc12*(W267A), which only cripples homotypic *Cdc12-Cdc12* interaction, others have shown that a gain-of-charge mutation in the nucleotide-binding P-loop (Figure S2A), *Cdc11*(G29D), although not sufficient to dissociate *Cdc11* from *Cdc12* *per se* (Nagaraj *et al.* 2008; Weems *et al.* 2014), is sufficient to make the G interface between *Cdc11* and *Cdc12* temperature sensitive for function [presumably because it interferes with propagation of GTP binding-induced conformational changes necessary for some aspect of proper septin architecture (Sirajuddin *et al.* 2009)]. The corresponding position in *Shs1* is G30. Clearly, survival of *CDC11-mC shs1Δ* cells required that *Cdc11* be able to form a proper G interface with *Cdc12* because, in the context of endogenously expressed *Cdc11-mC*, the G29D mutation was sufficient to cause inviability even at 30° (Figure 3A, compare second line to third line). Consistent with *Cdc11*(G29D) remaining bound to *Cdc12* (and thereby capping the ends of *Cdc12-Cdc3-Cdc10-Cdc10-Cdc3-Cdc12* heterohexamers in a nonfunctional manner), such cells remain inviable even in the absence of *Shs1* or in the presence of *Shs1*(G30D) (Figure 3A, compare second line to fourth and fifth lines). However, most tellingly and as predicted, in the absence of any competing *Cdc11*, crippling the G interface in *Shs1* with the G30D mutation is sufficient to rescue the viability of *cdc11Δ* cells (Figure 3A, compare sixth line to seventh line), just like a complete null (*shs1Δ*) (Figure 2). This finding suggests that this P-loop alteration is sufficient to dissociate *Shs1* from the ends of *Cdc12-Cdc3-Cdc10-Cdc10-Cdc3-Cdc12* heterohexamers, allowing for their polymerization by homotypic *Cdc12-Cdc12* interaction. Consistent with that view, presence of *Cdc12*(W267A) made *cdc11Δ shs1*(G30D) cells inviable (Figure 3A, compare seventh line to eighth line). These data provide strong evidence that in the cell, just as observed *in vitro* (Garcia *et al.* 2011), *Cdc11* and *Shs1* represent alternative terminal subunits in septin hetero-octamers. Moreover, as assessed in the context of one of the genetic backgrounds where *Shs1* function is required for viability (*cdc10Δ* cells at 25° on Gal medium) (Figure 1C), *Shs1*(G30D) was unable to support detectable growth, confirming that it is a loss-of-function allele (Figure 3B).

Assessment of the roles of the α0 helix and its basic residues in septin function

As revealed by the X-ray structure of human SEPT2, a short (approximately four-turn) α-helix (α0) lies just upstream of the P-loop (Sirajuddin *et al.* 2007) and likely does so in all septins (Figure S2A), although the N terminus (including the residues corresponding to their predicted α0) were deleted to obtain well-behaved crystals for other human septins (Zent *et al.* 2011; Macedo *et al.* 2013). A feature of α0 in human SEPT2 and most other septins is a prominent tract of basic residues (Figure S2A). It has been proposed that these positively charged residues are important for septin association with acidic PM phospholipids, especially PtdIns4,5P₂

Figure 2 Either *Shs1* or *Cdc11* can occupy the terminal position in septin hetero-octamers. (A) Left, strains of the indicated genotype (GFY-160, GFY-147, GFY-164, GFY-163, and GFY-437) harboring a *URA3*-marked *CEN* plasmid expressing WT *CDC11* were propagated, spotted and tested for ability to grow on medium lacking and containing 5-FOA to select for loss of the covering *CDC11*-expressing plasmid, as in Figure 1C. Right, scheme of septin constitution; *Cdc11*-capped octamers in gray and *Shs1*-capped octamers in purple. For clarity, the internal

subunits (*Cdc12*, *Cdc3*, and *Cdc10*) are colored according to the identity of the terminal subunit. Dashed circles, subunit that is absent; red asterisk, *cdc12-W267A* allele. (B) The top five strains from A were propagated twice on medium containing 5-FOA at 20° to select for cells lacking the covering plasmid, then samples of exponentially-growing cultures (20°) were examined by DIC microscopy. After this selection, the *cdc11Δ shs1Δ cdc12-W267A* triple mutant (not shown) was inviable. Arrowheads, cells with abnormal morphology.

(Zhang *et al.* 1999; Bertin *et al.* 2010). We perturbed this segment of *Shs1* in several ways and expressed the resulting mutant alleles from the chromosomal *SHS1* locus in our *Shs1*-dependent genetic backgrounds to assess their impact on *Shs1* function.

First, when the six Lys and Arg residues in the predicted $\alpha 0$ of *Shs1* were replaced with Ala, the residue that possesses the greatest α -helix-forming propensity (Myers *et al.* 1997), thus maintaining structure but eliminating charge, this mutant (designated *Shs1*-NT-A) was clearly nonfunctional because it was no longer able to support the growth of *cdc10Δ* cells at 25° on Gal medium (Figure 3B). We had previously observed that conversion of all four basic residues to Ala in $\alpha 0$ of *Cdc10* itself did not compromise its function under the same conditions (McMurray *et al.* 2011); however, upon revisiting this issue, we found that *Cdc10*-NT-A could not support growth at 30° or higher temperature (Figure S2B) and, even though it was well expressed and competent to be incorporated at the bud neck, it caused markedly aberrant cell morphology if present as the sole source of *Cdc10* when the cells were shifted to 30° (Figure S2C). It was somewhat surprising, therefore, that an N-terminal deletion ($\Delta 2$ -18) in *Shs1*, including most of its $\alpha 0$, was able to support growth of the *cdc10Δ* cells at 25° on Gal medium (Figure 3B). However, when the two remaining basic residues not removed by this deletion were mutated to Ala, the resulting *Shs1*($\Delta 2$ -18 K19A R20A) mutant, like the *Shs1*-NT-A mutant, was unable to support growth of the *cdc10Δ* cells at 25° on Gal medium (Figure S3A). These results dramatically highlight the importance of positive charge in this region for retention of *Shs1* (and *Cdc10*) function.

We then tested whether the same was true of the tract of basic residues in the $\alpha 0$ in *Cdc11*. As expected, when the six Lys and Arg residues in the predicted $\alpha 0$ of *Cdc11*-mC were replaced with Ala, this mutant (designated *Cdc11*-NT-A-mC) was clearly nonfunctional (Figure 3C, compare third line to first line). This behavior was not due to competition from *Shs1* because cells expressing *Cdc11*-NT-A-mC as the sole source of *Cdc11* were inviable even when *Shs1* was absent (Figure 3C, compare fourth line to third line) or lacked its own basic tract in $\alpha 0$ (Figure 3C, compare fifth line to third line). In agreement with other evidence that *Shs1*-NT-A is otherwise expressed, folded, and able to associate with *Cdc12*, it was capable, like WT *Shs1*, of blocking the growth

of *Cdc11*-deficient cells (Figure 3C, compare seventh line to sixth line). We were somewhat surprised, therefore, when we observed that a *Cdc11*($\Delta 2$ -18 R19A)-mC, a mutant that should mimic *Cdc11*-NT-A-mC with regard to its loss of positive charge, was able to support growth (Figure S3B, compare third line to first line). In this case, however, we suspected that the positive residues in *Shs1* might serve a partially redundant function for recruitment of septin complexes to the cytosolic side of the PM (Bertin *et al.* 2010; Bridges *et al.* 2014). Consistent with this view, cells expressing *Cdc11*($\Delta 2$ -18 R19A)-mC were inviable when *Shs1* was absent (Figure S3B, compare fourth line to third line) or carried the equivalent alterations in its N-terminal region (Figure S3B, compare fifth line to third line), even though *Shs1*($\Delta 2$ -18 K19A R20A) was produced, folded, and able to associate with *Cdc12*, because like WT *Shs1* or *Shs1*-NT-A, its expression blocked growth of *Cdc11*-deficient cells (Figure S3B, compare seventh line to sixth line).

One explanation for the apparent “rescue” of *Cdc11*($\Delta 2$ -18 R19A)-mC by the presence of *Shs1* (and its own set of basic residues) might be that at some frequency in normal septin structures, *Shs1* and *Cdc11* associate via an NC interface and that an intact N terminus in one subunit is sufficient to maintain that contact. This proposal is completely analogous to another, previously described situation where only one septin subunit need have an intact $\Delta \alpha 0$ to make a productive NC interface. Specifically, when expressed as the sole source of *Cdc10*, neither *Cdc10*($\Delta \alpha 0$) nor *Cdc10*(I22E), another mutant that disrupts a primary contact at the NC interface, was able to support growth; however, when coexpressed with WT *Cdc10*, *Cdc10*($\Delta \alpha 0$) was not toxic and was efficiently incorporated into the septin structures at the bud neck (McMurray *et al.* 2011). In agreement with this possibility, cells expressing *Cdc11*($\Delta 2$ -18) are viable in the presence of *Shs1*, but not when *Shs1* was absent or, most tellingly, when combined with *Shs1*($\Delta 2$ -18) (Figure 3D, compare fourth and fifth lines to third line), despite the fact that *Shs1*($\Delta 2$ -18) was produced, folded, and able to associate with *Cdc12* because its expression blocked growth of *Cdc11*-deficient cells (Figure S3, compare seventh line to sixth line). Also, it should be recalled that expression of *Shs1*($\Delta 2$ -18) in *cdc10Δ* cells (which have WT *CDC11*) allowed for growth at 25° on Gal medium (Figure 3B). Thus, as long as either of the

Figure 3 Characterization of Shs1 and Cdc11 alleles affecting their G and NC interfaces. (A) Strains of the indicated genotype, including the *cdc11(G29D)* and *shs1(G30D)* alleles, which alter a residue in the P-loop of the GTP-binding domain and harboring a plasmid expressing WT *CDC11* (GFY-58, GFY-164, GFY-121, GFY-308, GFY-375, GFY-147, GFY-397, and GFY-843), were propagated, spotted, and tested for ability to grow on medium either lacking or containing 5-FOA to select for loss of the covering *CDC11*-expressing plasmid, as in Figure 1C. (B) Strains of the indicated genotype expressing the indicated *shs1* alleles and harboring a plasmid expressing WT *CDC10* (GFY-87, GFY-137, GFY-369, GFY-249, and GFY-93), were tested as in Figure 1C. *shs1-NT-A* is Shs1 (R13A R14A K15A K16A K19A R20A); *shs1-α0Δ* is Shs1(Δ2-18). (C) Strains of the indicated genotype (GFY-58, GFY-164, GFY-246, GFY-247, GFY-307, GFY-147, and GFY-253) and harboring a plasmid expressing WT *CDC11* were tested as in A. *cdc11-NT-A* is Cdc11(R12A K13A R14A K15A K18A R19A). (D) Strains of the indicated genotype and harboring a plasmid expressing WT *CDC11* (GFY-58, GFY-164, GFY-123, GFY-165, GFY-161, GFY-147, and GFY-148) were tested as in A. (E) Exponentially

growing cultures of strains (GFY-160, GFY-161, GFY-375, and GFY-307) co-expressing, respectively, mCherry- and eGFP-tagged versions of either WT Cdc11 and Shs1, or the indicated mutant versions (as well as a plasmid-born copy of untagged WT *CDC11* to maintain cell viability), were examined by fluorescence microscopy. Dotted white line, cell periphery.

two alternative terminal subunits retained an intact $\alpha 0$, cells were viable. Collectively, these results provide genetic evidence that heterotypic NC junctions between Cdc11 and Shs1 may occur *in vivo*.

To obtain additional insight about their properties, cells coexpressing paired alleles of both Cdc11 (tagged with mC) and Shs1 (tagged with eGFP) from the corresponding chromosomal loci were propagated in the presence of a *CEN* plasmid expressing untagged *CDC11* (required for their viability) and examined by fluorescence microscopy. Cdc11($\Delta\alpha 0$) and Shs1($\Delta\alpha 0$) presumably have a defective NC interface and will be unable to interact in that mode. However, they each have an intact G interface and retain, collectively, 3 of their 12 Lys and Arg residues. Hence, we expected that they would compete well with the untagged Cdc11 for association with Cdc12 and become incorporated into the septin structures at the bud neck, as observed (Figure 3E). Conversely, for Cdc11(G29D) and Shs1(G30D), which have a crippled G interface, elevated cytoplasmic fluorescence was observed, suggesting that they competed less well with untagged Cdc11 for incorporation at the bud neck, as anticipated (Figure 3E). Strikingly, retention of $\alpha 0$, but substitution of all 12 Lys and Arg residues in Cdc11 and Shs1 combined, caused the most severe reduction in the efficiency of their incorporation at the bud neck (Figure 3E). This latter finding suggests that recruitment of Cdc11- and Shs1-containing complexes to the cytosolic side of the PM is a prelude to their assembly into filaments and higher-order structures *in vivo*, consistent with recent imaging studies *in vivo* (Bridges *et al.* 2014) and with *in vitro* studies wherein association with lipid monolayers

promoted septin filament formation (Bertin *et al.* 2010). Thus, the importance of positive charge in $\alpha 0$, at least for Cdc11 and Shs1 (and, likely, Cdc10; Figure S2) (Figures 3 and Figure S3), seems primarily to be in concentrating septin complexes at the PM, thereby increasing the rate of their incorporation into polymers.

The CTE of Cdc11 and Shs1 share a role critical for optimal septin function

One feature conserved throughout the phylogeny of the septin protein family in opisthokont eukaryotes, from fungi to humans (Pan *et al.* 2007; Nishihama *et al.* 2011; Hall and Russell 2012; Fung *et al.* 2014), is the presence of a long CTE appended to the globular Ras-related GTP-binding domain (Sirajuddin *et al.* 2007; Weirich *et al.* 2008). As first recognized in sequence comparisons of an *Aspergillus nidulans* septin (AspB) to other septin orthologs (Momany and Hamer 1997), the CTE always contains a segment of significant length wherein the spacing of overtly hydrophobic residues (and occasional polar residues whose arms are of sufficient length to have significant hydrophobicity) matches the 4–3 repeat characteristic of sequences able to form either homotypic or heterotypic coiled coils (Harbury *et al.* 1995; Parry *et al.* 2008). Of the five mitotically expressed *S. cerevisiae* septins, Cdc10 is the only subunit that lacks such a CTE. Genetic analysis and *in vitro* biochemical studies have demonstrated that the CTEs of Cdc3 and Cdc12 are essential for cell viability (Versele *et al.* 2004), needed for high-affinity Cdc3–Cdc12 pairing (presumably via an NC interface) when these subunits are coexpressed as recombinant

proteins in bacteria (Versele *et al.* 2004), and required *in vitro* for paired filament formation in solution when *Cdc3*, *Cdc10*, *Cdc11*, and *Cdc12* are coexpressed (Bertin *et al.* 2010). Moreover, although not necessary for assembly of hetero-octamers *per se*, in the absence of the CTEs of *Cdc3* and *Cdc12*, the hetero-octamers formed are markedly less stable than WT hetero-octamers (Bertin *et al.* 2010). On the basis of such evidence, and ultrastructure analysis by electron microscopy (Bertin *et al.* 2008), it appears that the CTEs of *Cdc3* and *Cdc12* form a parallel heterotypic coiled coil projecting orthogonal to the hetero-octameric rod, which associates in register with the same coiled coil in a laterally adjacent hetero-octamer, forming an antiparallel four-helix bundle whose formation promotes highly cooperative polymerization of hetero-octamers into paired filaments. Thus, at least in the long paired filaments formed from purified recombinant *Cdc11*–*Cdc12*–*Cdc3*–*Cdc10*–*Cdc10*–*Cdc3*–*Cdc12*–*Cdc11* hetero-octamers in solution, the coiled coil formed between the CTEs of *Cdc3* and *Cdc12* serves the same role as the rungs in a ladder.

By contrast, the CTE of *Cdc11* is not required for stable hetero-octamer formation (Versele *et al.* 2004; Bertin *et al.* 2008) or for the formation of long-paired filaments *in vitro* (Bertin *et al.* 2008, 2010), and cells expressing *Cdc11* lacking its CTE are viable, although they do grow with markedly elongated buds and display a defect in cytokinesis (Versele *et al.* 2004). Our observations that fusion of mC to the CTE of *Cdc11* sensitized growth of the cells to the absence of *Shs1* (Figure 1A), and that removal of the CTE of *Cdc11* in the same context made cell viability completely dependent on the presence of *Shs1* (Figure 1B), suggested that the CTEs of *Cdc11* and *Shs1* might have a common function. The latter sensitized strain, as well as the three other genetic backgrounds we devised that also depend on *Shs1* expression for growth, provided the means for detailed genetic analysis of the CTE of *Shs1*.

Indeed, just like a null mutation (*shs1*Δ), a derivative of *Shs1* lacking its CTE (Δ349–551) (Figure S4A), designated *shs1*(ΔCTE), and a derivative in which only its predicted coiled-coil-forming segment (Δ465–496) (Figure S4A) was deleted, designated *shs1*(ΔCC), were unable to support the growth of *cdc10*Δ cells at 25° on Gal medium (Figure 4A). Likewise, and just like an *shs1*Δ mutation, *shs1*(ΔCTE) was unable to support the growth of cells expressing *Cdc11* (ΔCTE)–mC as the sole source of *Cdc11* (Figure 4B). Even an otherwise normal *Shs1* in which just 10 side chains in the center of the predicted coiled-coil-forming segment (Figure 4C) were mutated to Ala, designated *shs1*–CC-A, was unable to support the growth of the *cdc10*Δ tester cells (Figure 4A). The inability of *shs1*(ΔCTE) to function was not due to lack of expression because it was able to prevent the growth of cells lacking *cdc11*Δ (Figure 4B) and because, when tagged with eGFP and expressed from its chromosomal locus in the presence of a *CEN* plasmid expressing untagged *CDC11* (required for viability) and examined by fluorescence microscopy, *shs1*(ΔCTE) (and *shs1*–CC-A) were efficiently incorporated

at the bud neck, as expected for a properly folded septin subunit (Figure 4D). Interestingly, when 12 side chains in the C-terminal two-thirds of the predicted coiled-coil-forming segment in *Cdc11* (Figure 4C) were mutated to Ala in *Cdc11*–mC, it was able to support the growth of cells lacking *Shs1* or expressing *Shs1*–CC-A (Figure 4E), unlike *Cdc11*(ΔCTE)–mC (Figure 1C and Figure 4B). Thus, the function of the CTE of *Cdc11* was preserved in *Cdc11*–CC-A, which implicates the upstream one-third of the predicted coiled-coil-forming segment as the most critical for its physiological role, consistent with the fact that this portion of the coiled coil is somewhat more highly conserved than the portion we mutated (Figure 4C).

Predicted coiled-coil region is the critical functional element in *Shs1* and *Cdc11* CTE

Compared to its predicted coiled-coil-forming segment (CC element), the remainder of the CTE in both *Shs1* and *Cdc11* is very poorly conserved (Figure S4, A and B). Yet, the CC element in *Shs1* is always situated about ~50–150 residues from its C terminus, whereas that in *Cdc11* is situated right at its C terminus. To determine whether the position of the CC element relative to the body of the globular GTP-binding domain, and the difference in its location between *Shs1* and *Cdc11*, was important for its physiological function, we generated deletions that removed various segments of the intervening “spacer” sequence, or the entire spacer, between the end of α6 in the G domain and the CC element in *Shs1* and *Cdc11* (Figure S4, A and B), and tested their ability to complement.

Unlike absence of *Shs1*, or removal of its entire CTE (including its CC element), versions of *Shs1* retaining the CC element, but lacking any segment, or the entirety, of the intervening spacer sequence were all able to support the growth of *cdc10*Δ cells on Gal medium at 25° (Figure 5A). Likewise, in contrast to *Cdc11*(ΔCTE)–mC, which is unable to support the growth of *shs1*Δ cells, versions of *Cdc11* retaining the CC element, but lacking any segment, or the entirety, of the intervening spacer sequence were all able to support the growth of *shs1*Δ cells (Figure 5B). Moreover, unlike the inviability of any cell that expressed versions of both *Cdc11* and *Shs1* that lack their CTEs (Figures 4B and Figure S5), cells expressing versions of *Cdc11* and *Shs1* that each retain only their CC elements grew robustly (Figure 5C).

This remarkable plasticity was in stark contrast to the outcome of similar deletion analysis of the spacer sequences between α6 and the CC elements in the CTEs of *Cdc3* and *Cdc12*. The spacer in *Cdc3* shows a very high degree of conservation in both sequence and length (Figure S4C). As anticipated, deletion of even as few as 12 residues of the spacer sequence in *Cdc3* abrogated its ability to complement *Cdc3*-deficient cells, just like a null allele (*cdc3*Δ), deletion of its entire CTE, or replacement of 10 residues of its predicted CC element with Ala (Figure 5D). Similarly, in *Cdc12*, only removal of the most upstream and least conserved 12-residue segment of its spacer (Figure S4D) preserved function, whereas all other perturbations of the CTE of *Cdc12*, including replacement of 12 residues of its predicted CC

Figure 4 CTEs of Shs1 and Cdc11 are required for septin function, but not for incorporation at the bud neck. (A) Strains expressing the indicated *shs1* alleles (GFY-87, GFY-137, GFY-94, GFY-98, and GFY-178) were analyzed as in Figure 3B. *shs1*(ΔCTE), *Shs1*(Δ349–551); *shs1*(ΔCC), (Δ465–496); *shs1*–CC-A, *Shs1*(Y465A R468A I472A R475A L479A L482A S486A E489A R493A E496A). (B) Strains expressing the indicated *shs1* and *cdc11* alleles (GFY-58, GFY-164, GFY-122, GFY-166, GFY-162, GFY-147, and GFY-149) were analyzed as in Figure 1, A and C. (C) Sequence alignment using CLUSTAL-W (Thompson *et al.* 1994) of the C-terminal coiled-coil-containing regions of the CTEs in the *SHS1* (top) and *CDC11* (bottom) homologs from *S. cerevisiae* (Sc), *Lachancea thermotolerans* (Lt), *A. gossypii* (Ag), *Vanderwaltozyma polyspora* (Vp), *Zygosaccharomyces rouxii* (Zr), *Kluyveromyces lactis* (Kl), *C. glabrata* (Cg), *C. albicans* (Ca), and *Debaryomyces hansenii* (Dh) (residue numbers in parentheses). White letters on black boxes, invariant residues; red asterisks, hydrophobic residues (or residues with hydrophobic arms) in the 4–3 spacing predicted for the coiled-coil-forming segment (CC) of the

CTE, which were mutated to Ala the CC-A mutants. (D) Cells coexpressing an mCherry-tagged derivative of either WT Cdc11 or the indicated mutant and an eGFP-tagged derivative of either WT Shs1 or the indicated mutant (GFY-160, GFY-162, and GFY-475), and also harboring a plasmid expressing untagged WT *CDC11* to maintain viability, were examined by fluorescence microscopy. Dotted white line, cell periphery. (E) Strains expressing the indicated *CDC11* and *SHS1* alleles (GFY-58, GFY-164, GFY-378, GFY-383, and GFY-475) were analyzed as in B. *cdc11*–CC-A, Cdc11(E377A V380A L384A K387A L391A R394A L398A I401A L405A E408A K412A E415A).

element with Ala, were either severely crippled or totally ablated in their ability to complement *Cdc12*-deficient cells (Figure 5E), despite the fact that even complete truncation of their CTEs did not prevent either *Cdc3* or *Cdc12* expression or incorporation at the bud neck (Figure 5F).

For *Shs1*, unlike the total loss of function caused by replacement of 10 residues of the 4–3 repeat in its CC element (Figure 4A), replacement of no single 1 of these 10 residues was sufficient to abolish *Shs1* function. We noted, however, that reducing the hydrophobicity at three such sites—Y465A at the start of the CC element, and nearby L479A and L482 (Figure 4C and Figure S4A)—clearly compromised *Shs1* function (Figure 5G). The context in which these residues lie in *Shs1* (Y-x₂-R-x-E-x-I-x₆-LEEL) is related to a similar motif (Y-x₂-R-x-E-x-I-x-LEEE) found in the N-terminal portion of the CC element of *Cdc11* (Figure 4C and Figure S4B). Although replacement of 12 residues of the 4–3 repeat downstream of this motif in the CC element of *Cdc11* did not destroy its function, as judged by its ability to support the growth *shs1*Δ cells (Figure 4E), a *Cdc11*(Y369A E363A I366A E370A L373A) mutant was unable to (data not shown). Thus, the N-terminal residues of the CC element are those most critical for its physiological function. Moreover, replacement of conserved residues in the CC elements of both *Shs1* and *Cdc11* with α-helix-breaking Pro residues [*Shs1*(K478P L479P), *Shs1*(S486P A487P), *Cdc11*(E363P E364P), *Cdc11*(E369P E370P), *Cdc11*(R379P V380P), and *Cdc11*(K387P R388P)] also destroyed CTE function (data not shown).

The CTEs of Cdc11 and Shs1 can function in trans

Given the apparent dispensability of the spacer regions for CTE function in both *Cdc11* and *Shs1*, we also wondered whether CTE function would be sufficiently flexible to allow them to operate if swapped onto the other terminal subunit within otherwise normal septin complexes. First, however, we had to rule out that a *Cdc11*(ΔCTE)–CTE^{*Shs1*} chimera did not merely act like WT *Cdc11* and, likewise, that a *Shs1*(ΔCTE)–CTE^{*Cdc11*} chimera did not simply act like WT *Shs1*, which, if true, would confound interpretation of the results of a CTE swap. Fortunately, two of our sensitized genetic backgrounds allowed us to address this question directly.

First, as shown earlier and recapitulated in this context, *shs1*Δ cells expressing *Cdc11*–mC survive, whereas *shs1*Δ cells expressing *Cdc11*(ΔCTE)–mC do not (Figure 6A, compare line two to line one). Hence, these cells serve as reporters of *Cdc11* CTE function. In this situation, expression of *Cdc11*(ΔCTE)–CTE^{*Shs1*} as the sole source of *Cdc11* did not support growth (Figure 6A, line three). Thus, the *Cdc11*(ΔCTE)–CTE^{*Shs1*} chimera did not simply behave like WT *Cdc11*. Second, as shown earlier and recapitulated in this context, *cdc10*Δ cells expressing *Shs1* are viable at 25° on Gal medium, whereas *cdc10*Δ cells lacking *Shs1* (Figure 6B, compare line two to line one) or expressing *Shs1*(ΔCTE) (Figure 4A) are dead. Hence, these cells serve as reporters of *Shs1* CTE function. Under these conditions, expression of *Shs1*(ΔCTE)–CTE^{*Cdc11*} as the sole source of *Shs1* did not support growth (Figure 6B, line three). Thus, the *Shs1*(ΔCTE)–CTE^{*Cdc11*} chimera did not simply behave like WT *Shs1*. Hence, it was now

Figure 5 Mutational analysis of the spacer region that separates the globular G domain from the CC element in the CTEs of Shs1, Cdc11, Cdc3, and Cdc12. (A) Strains expressing the indicated *shs1* alleles C terminally tagged with eGFP (GFY-87, GFY-137, GFY-94, GFY-233, GFY-234, GFY-235, GFY-236, GFY-237, GFY-519, GFY-503, and GFY-514) were analyzed as in Figure 2B. (B) Strains expressing the indicated *cdc11* alleles C terminally tagged with mCherry (GFY-164, GFY-166, GFY-381, GFY-398, GFY-399, GFY-382, GFY-513, GFY-524, and GFY-512) were analyzed as in Figure 3A. (C) Strains coexpressing the indicated *CDC11* and *SHS1* alleles (GFY-58 and GFY-676) were analyzed as in B. (D) Strains expressing the indicated *cdc3* alleles C terminally tagged with mCherry (GFY-126, YMV633, GFY-764, GFY-501, GFY-494, GFY-493, GFY-507, and GFY-511) were analyzed for ability to restore viability to *cdc3Δ* cells. (E) Strains expressing the indicated *cdc12* alleles C terminally tagged with mCherry (GFY-251, YMV61, GFY-515, GFY-535, GFY-516, GFY-498, GFY-510, and GFY-525) were analyzed for ability to restore viability to *cdc12Δ* cells. (F) Exponentially growing cells from (D) and (E), harboring plasmids expressing, respectively, WT untagged *CDC3* or *CDC12* to maintain viability, were visualized by fluorescent microscopy. Dotted white line, cell periphery. (G) Strains expressing the indicated *shs1* alleles C terminally tagged with eGFP (GFY-87, GFY-137, GFY-179, GFY-180, GFY-181, GFY-362, GFY-368, GFY-366, GFY-367, GFY-363, GFY-391, and GFY-370) were analyzed as in A.

possible to properly interpret the outcome of expression of these chimeras in cells that otherwise possess the capacity to assemble both *Cdc11*- and *Shs1*-capped hetero-octamers (Figure 6C).

As demonstrated earlier (Figure 4B) and recapitulated in this context, when both *Cdc11* and *Shs1* lack their CTEs, cells are inviable (Figure 6A, compare line five to line four). Strikingly, however, expression of *Shs1(ΔCTE)*–CTE^{*Cdc11*} as the sole source of *Shs1* in cells expressing *Cdc11(ΔCTE)* permitted growth (Figure 6A, line six), and, conversely, expression of *Cdc11(ΔCTE)*–CTE^{*Shs1*} as the sole source of *Cdc11* in cells expressing *Shs1(ΔCTE)* supported growth (Figure 6A, line seven). Thus, when incorporated at their terminal position in septin hetero-octamers, the CTEs of *Shs1* and *Cdc11* must share at least one function that is essential for cell survival, presumably the recruitment of another (nonseptin) protein.

We noted, however, that cells coexpressing *Cdc11(ΔCTE)*–CTE^{*Shs1*} and *Shs1(ΔCTE)*–CTE^{*Cdc11*} grew somewhat more

robustly (Figure 6A, line eight), suggesting that perhaps the single chimeras were not as efficiently expressed or assembled into complexes as when they were coexpressed. However, examination of the cells expressing fluorescently tagged versions of these molecules showed that each was expressed equivalently and well incorporated at the bud neck (Figure 6D), regardless of the status of the other subunit.

Another explanation for the more robust growth of cells coexpressing *Cdc11(ΔCTE)*–CTE^{*Shs1*} and *Shs1(ΔCTE)*–CTE^{*Cdc11*} could simply represent an avidity effect, namely that any cellular factor will have a high likelihood of binding if it encounters two CTE in the same immediate vicinity rather than one (Figure 6C). As one means to address the latter possibility, we took advantage of the fact that at another position in hetero-octamers, two subunits are in close proximity, namely the *Cdc10*–*Cdc10* NC dimer at the center of the rod (Bertin *et al.* 2008). We therefore constructed versions of *Cdc10* alone, or with the CTE of either *Shs1* or

Figure 6 Swap of the CTEs of Shs1 and Cdc11 retains their function, but does not confer a shift in subunit identity. (A) Strains expressing the indicated Shs1 and Cdc11 derivatives, C terminally tagged with eGFP and mCherry, respectively (GFY-164, GFY-166, GFY-544, GFY-160, GFY-162, GFY-540, GFY-542, and GFY-692), were analyzed as in Figure 3A. (B) Strains expressing the indicated *shs1* alleles (GFY-87, GFY-137, and GFY-548) were analyzed as in Figure 3B. (C) As in Figure 2A, scheme of the septin subunit composition of five yeast strains, numbered 1–5 on the right in A, of the indicated genotypes. Gray, Cdc11-capped octamers; purple, Shs1 capped octamers; solid line, CTE; cylinder, CC element; empty cylinder with dotted lines, deleted CTE. For clarity, the CTEs of Cdc3 and Cdc12 are not shown. (D) Strains expressing the indicated derivatives of Cdc11 (left) and Shs1 (right) (GFY-540, GFY-542, and GFY-692) were visualized by fluorescence microscopy. Dotted white line, cell periphery. (E) A strain (GFY-162) coexpressing Cdc11(Δ357–415) and Shs1(Δ349–551) as the sole source of these two subunits, and covered with a *URA3*-marked *CEN* plasmid expressing WT untagged *CDC11* to maintain viability, was transformed with plasmids expressing, as indicated, either a Cdc10–CTE^{Shs1} chimera (pGF-IVL370) or a Cdc10–CTE^{Cdc11} chimera (pGF-IVL371), and with appropriate control vectors (pGF-IVL1, pRS315 and pGF-preIVL6). Overnight culture on SD–Ura–Leu was used to select for plasmid maintenance before spotting onto SD–Leu in the absence (left) and presence (right) of 5-FOA to select for loss of the covering *CDC11* plasmid.

ity, was transformed with plasmids expressing, as indicated, either a Cdc10–CTE^{Shs1} chimera (pGF-IVL370) or a Cdc10–CTE^{Cdc11} chimera (pGF-IVL371), and with appropriate control vectors (pGF-IVL1, pRS315 and pGF-preIVL6). Overnight culture on SD–Ura–Leu was used to select for plasmid maintenance before spotting onto SD–Leu in the absence (left) and presence (right) of 5-FOA to select for loss of the covering *CDC11* plasmid.

Cdc11 appended, each tagged on its C terminus with mC, and confirmed that all of them were able rescue the growth of *cdc10Δ* cells at 30° on glucose medium (data not shown). We found that even a higher than endogenous level of expression (from a *CEN* plasmid) of neither Cdc10–CTE^{Shs1} nor Cdc10–CTE^{Cdc11} was able to rescue the growth of *cdc11(ΔCTE) shs1(ΔCTE)* cells (Figure 6E). Thus, to carry out their physiological role the CTEs of Cdc11 and Shs1 must be positioned at the ends of septin hetero-octamers.

Finally, it has been reported that, when expressed from a plasmid as a GFP-tagged protein, the Shs1 ortholog of *A. gossypii* is efficiently incorporated into the septin collar at the bud neck in both WT and *shs1Δ* *S. cerevisiae* cells, and that expression of WT AgShs1 in either WT or *shs1Δ* *S. cerevisiae* is innocuous, whereas AgShs1(ΔCTE)–GFP, and much less so AgShs1(ΔCC)–GFP, caused *shs1Δ* cells (but not WT cells) to assume a markedly aberrant cell morphology (Meseroll *et al.* 2012). These findings are at odds with our demonstration here that the CC element in the Shs1 CTE is the segment that mediates its physiological function. For this reason, we revisited the ability of AgShs1 to substitute for ScShs1 using our well-defined Shs1-dependent genetic backgrounds. We found, first, and contrary to the other study (Meseroll *et al.* 2012), that AgShs1 expressed from the chromosomal *SHS1* locus is not functional in *S. cerevisiae* because, unlike authentic ScShs1 expressed in the identical manner, it

did not support the growth of either *cdc10Δ* cells at 25° on Gal medium (Figure S6A, top) or the growth of *Cdc11(ΔCTE)–mC* cells at 30° on glucose medium (Figure S6A, middle). Yet, AgShs1 was stably expressed and was clearly able to associate with Cdc12 as judged by two criteria, its ability to kill *cdc11Δ* cells (Figure S6A, bottom) and its efficient incorporation at the bud neck as seen by fluorescence microscopy (Figure S6B). Strikingly, unlike AgShs1 itself, a ScShs1(ΔCTE)–CTE^{AgShs1} chimera was able to support growth of both of our Shs1-dependent reporter strains (Figure S6A, top and middle), in keeping with the very high degree of sequence conservation between the CC elements in ScShs1 and AgShs1 (Figure S6C). Moreover, the inverse chimera, AgShs1(ΔCTE)–CTE^{ScShs1}, was unable to support the growth of either Shs1-dependent reporter strain (Figure S6A, top and middle), indicating that it is the G domain of AgShs1 that is incompatible with proper function in *S. cerevisiae*.

Discussion

To analyze in an incisive manner the elements and residues in Shs1 required for its contributions to septin function *in vivo*, we exploited four different conditions under which expression of wild-type Shs1 is essential for cell viability. Among the manipulations used was attachment of fluorescent tags to the C terminus of either Cdc11 or Shs1, which we noted

compromises the function of these septins to at least some degree (Figure S5), but this modest debility did not otherwise influence or alter our primary conclusions. Indeed, the same phenotype was uniformly observed whenever an *Shs1* mutant was tested in each of the four sensitized genetic backgrounds, which yielded considerable new information not previously known about this septin subunit. Furthermore, because *Shs1* and *Cdc11* are alternative terminal subunits of septin heterooctamers (Garcia *et al.* 2011) and clearly compete *in vivo* for occupancy of that position (McMurray *et al.* 2011), our analysis had to take the contributions of *Cdc11* into account and, in the process, also revealed considerable new insights about the relative contributions of, and the interplay between, these two subunits.

Phosphorylation of *Shs1* within its CTE is not required for function

During passage through the cell cycle, septins are decorated with a variety of chemical marks, including phosphorylation, SUMOylation, ubiquitinylation, and both N α - and N ϵ -Lys acetylation (McMurray and Thorner 2009; Hernandez-Rodriguez and Momany 2012). The extensive modification of septin structures has been likened to that of histones in chromatin (McMurray and Thorner 2009). In particular, phosphorylation events have been implicated in septin assembly, disassembly, and/or function. For example, it has been reported that phosphorylation of *Cdc3* by *Cdc28* promotes (old) ring dissolution (Tang and Reed 2002) and that phosphorylation of *Cdc10* by *Cla4* promotes septin filament formation (Schmidt *et al.* 2003; Versele and Thorner 2004). For *Shs1*, its dephosphorylation by the Rts1-bound form of phosphoprotein phosphatase 2A is purportedly necessary for split ring disassembly at the end of cytokinesis (Dobbelaere *et al.* 2003). We noted, however, that many of the phosphorylation sites in *Shs1*, either proposed (Dobbelaere *et al.* 2003) or mapped after exhaustive *in vitro* phosphorylation by CDKs (*Cdc28* and *Pho85*) or *Gin4* (Egelhofer *et al.* 2008), fall within a very poorly conserved segment of the *Shs1* CTE, the “spacer” that connects the globular GTP-binding domain to the very well conserved coiled-coil-forming sequence. In keeping with this poor conservation, our genetic analysis revealed that substitution of all 19 of the proposed or mapped phosphorylation sites in the CTE with Ala did not detectably impair *Shs1* function (Figure 1D, E and F). Moreover, removal of the entire spacer region (deletion of residues 342–436) also had no deleterious effect on the ability of *Shs1* to support cell growth (Figure 5A), eliminating any other potential phosphorylation sites in the CTE that we did not mutate from being critical in control of *Shs1*. Thus, cycles of phosphorylation and dephosphorylation of the CTE can have no significant role in regulating the function of this septin. Similarly, in the filamentous fungus *A. gossypii*, its *Shs1* ortholog is also multiply phosphorylated within its CTE near its predicted coiled coil domain, and two protein kinases (the orthologs of *Elm1* and *Gin4*) have been implicated in septin ring assembly in this organism; yet, unlike loss of the kinases, a *shs1*–9A allele did

not display any marked defect in septin ring assembly or dynamics (Meseroll *et al.* 2012).

In contrast to elimination of a phosphorylation site, mutation to a negatively charged residue in an attempt to mimic persistent phosphorylation is fraught with the potential for nonspecific effects on the permanent electrostatics of the mutated region of the protein. Remarkably, however, even conversion of 13 sites to Asp in the *Shs1* CTE was tolerated in two different *Shs1*-dependent backgrounds (Figure 1, E and F). Revealing, however, the dramatic change to the charge of the *Shs1* CTE was not tolerated in cells lacking *Cdc10* (Figure 1D). This observation alerted us to the possibility that one role of the *Shs1* CTE may be shared, in part, with a function also carried out by *Cdc10* (see accompanying article, Finnigan *et al.* 2015). On the other hand, our *in vivo* studies also highlight the difficulty in interpreting the effects of phosphomimetic mutations observed *in vitro*. For example, purified *Shs1*-capped heterooctamers form elaborate spirals, rings, and “bird nest” structures *in vitro*, whereas heterooctamers capped with *Shs1*(S259D) form instead a meshwork-like structure with a regularly repeating pattern (Garcia *et al.* 2011). Such a gauze-like arrangement of filaments (attributed to septins by antibody decoration) was observed at the bud neck when replicas of fixed yeast spheroplasts were examined after “unroofing” by freeze fraction and deep etching (Rodal *et al.* 2005) and lattices of crisscrossed filaments thought to be septins have been seen in more recent EM analysis of the structures assembled at the bud neck (Bertin *et al.* 2012; Ong *et al.* 2014). However, when present as the sole source of *Shs1*, neither *Shs1*(S259A) nor *Shs1*(S259D) had any effect on the ability of *Shs1* to support growth in three different *Shs1*-dependent strain backgrounds (Figure 1, D–F), and otherwise wild-type cells expressing either *Shs1*(S259A) or *Shs1*(S259D) as the sole source of this subunit have a normal morphology (data not shown). A difficulty in extending the results of *in vitro* experiments performed with only *Shs1*-capped heterooctamers to a cellular context is, of course, that competing *Cdc11* is present. In fact, as we demonstrated previously (McMurray *et al.* 2011) and amply confirmed here, the lethality of a *cdc11* Δ strain arises from “capping” of *Cdc12*–*Cdc3*–*Cdc10*–*Cdc10*–*Cdc3*–*Cdc12* heterohexamers with *Shs1*. Indeed, yeast expressing only *Shs1* as the sole terminal subunit, but are otherwise wild type, are inviable (Figure 2A). Furthermore, as long as its G interface is intact, overexpression of any *Shs1* allele is able to outcompete the endogenous level of *Cdc11* and is toxic to cells (Iwase *et al.* 2007; data not shown). Taken together, our data emphasize an important distinction between identification of sites of post-translational modification in a septin and the care needed to assess their actual contribution to the physiological function of that septin *in vivo*.

Evidence for heterotypic octamer-octamer interaction *in vivo*

Our results revealed a number of important nuances about the two terminal subunits. Although GTP binding is critical

for septin-septin association via their G interface (Versele and Thorner 2004; Sirajuddin *et al.* 2007; Nagaraj *et al.* 2008; Sirajuddin *et al.* 2009; Weems *et al.* 2014), our results suggest that the behavior of *Cdc11* and *Shs1* mutants defective in GTP binding is also influenced by their capacity to form an NC interface with an adjacent octamer (Figure 7). Past work (Bertin *et al.* 2008; Garcia *et al.* 2011; McMurray *et al.* 2011) indicates that *Cdc11*–*Cdc11* interaction via their NC interface occurs *in vitro* and *in vivo* (and is responsible for filament formation), whereas *Shs1*–*Shs1* NC interaction does not occur either *in vitro* or *in vivo* (and lacks the capacity to mediate filament formation). Although a P-loop mutation (G29D) in the GTP-binding pocket of *Cdc11* clearly causes a partial loss of septin function (Nagaraj *et al.* 2008; Weems *et al.* 2014), it does not displace *Cdc11* from *Cdc12* (Figure 3, A and E). In this regard, it has been shown that a septin-septin G interface can influence the neighboring septin-septin NC interface (Sirajuddin *et al.* 2009). We speculate, therefore, that the converse might also be true. Because, in a filament, each *Cdc11* engages in an NC interface with a *Cdc11* in a neighboring hetero-octamer, it may provide additional stabilization, perhaps explaining why *Cdc11*(G29D) remains bound. By contrast, a corresponding P-loop mutation (G30D) in the GTP-binding pocket of *Shs1* renders it unable to associate with *Cdc12*, as judged by its inability to prevent growth of a *cdc11Δ shs1Δ* double mutant (Figure 3A). There is no evidence that a *Shs1*–*Shs1* NC interface ever exists either *in vitro* (Garcia *et al.* 2011) or *in vivo* (McMurray *et al.* 2011), perhaps explaining why *Shs1*(G30D) is able to dissociate from the hetero-octamer. Alternatively, our observations may simply reflect that these P-loop mutations destabilize the *Shs1*–*Cdc12* G interface to a much greater extent than the *Cdc11*–*Cdc12* G interface. Strikingly, however, when coexpressed with either WT *CDC11* or *Cdc11*(G29D), *Shs1*(G30D) localized strongly at the bud neck (Figure 3E). The most parsimonious interpretation of these observations is that heterotypic *Shs1*–*Cdc11* NC interactions do occur and, thus, that *Shs1*-capped hetero-octamers must interact to a substantial degree with *Cdc11*-capped hetero-octamers (Figure 7).

We used perturbations of the NC interface in *Cdc11* and in *Shs1* to address this issue more directly. Prior work demonstrated that a *Cdc11* mutant lacking its $\alpha 0$, which contributes some contacts that form the NC interface (Sirajuddin *et al.* 2007), is able to assemble into *Cdc11*-capped hetero-octamers, but the resulting hetero-octamers are unable to polymerize into filaments *in vitro* (Bertin *et al.* 2008) and, correspondingly, that cells expressing *Cdc11*($\Delta\alpha 0$) in the absence of *SHS1* are also unable to form septin filaments and are inviable (McMurray *et al.* 2011). However, as we showed here, with *Shs1* present, cells expressing *Cdc11*($\Delta\alpha 0$) as the sole source of *Cdc11* were viable and, likewise, *CDC11 shs1*($\Delta\alpha 0$) were viable, whereas only the *cdc11*($\Delta\alpha 0$) *shs1*($\Delta\alpha 0$) double mutant was inviable (Figure 3). Again, these data are most compatible with the idea that as long as an intact NC surface is present in either *Cdc11* or *Shs1*, a sufficiently stable heterotypic *Cdc11*–*Shs1* NC interaction can

Figure 7 Relationships between and roles of *Cdc11* and *Shs1* in septin hetero-octamers. Top: our findings suggest the indicated overall model for the organization of *Cdc11*- and *Shs1*-capped hetero-octamers in the higher-order septin structures assembled at the bud neck. Bottom: schematic summary of the experimental basis of certain of our major conclusions. (1) The spacer that tethers the CC element in the CTE to the globular GTP-binding domain is not required for the function of either *Cdc11* or *Shs1*. (2) In contrast to the terminal subunits, the spacer that tethers the CC element in the CTE to the globular GTP-binding domain is critical for the function of both *Cdc3* or *Cdc12*. (3) The CTEs of *Cdc11* and *Shs1* CTE can function *in trans* when situated at the termini of hetero-octamers, but not when situated internally in hetero-octamers (attached to *Cdc10*). (4) Several of our observations provide genetic evidence that in addition to the well-characterized homotypic *Cdc11*–*Cdc11* NC interface contacts responsible for filament polymerization that heterotypic *Cdc11*–*Shs1* NC junctions are also present and necessary for the physiological function(s) of septin-containing structures.

occur (Figure 7). Likewise, *Cdc10*($\Delta\alpha 0$) can form a stable NC interface with WT *Cdc10*, as judged by its efficient incorporation into the septin structures at the bud neck (McMurray *et al.* 2011). This precedent also extends to the G interface; in *Cdc12*, mutation of a hydrophobic residue at its G contact surface (W267A) does not prevent heterotypic *Cdc12*–*Cdc11* G interface interaction, but does block homotypic *Cdc12*–*Cdc12* G interface interaction (McMurray *et al.* 2011), as we confirmed (Figure 2A). Thus, if homotypic *Shs1*–*Shs1* NC interaction were the sole NC association mode for this subunit and crucial for its function, then one might expect that *CDC11 shs1*($\Delta\alpha 0$) cells would display a growth defect [e.g., when *shs1*($\Delta\alpha 0$) was tested in the *cdc10Δ* strain, which contains WT *CDC11*], but this was not the case (Figure 3B). Similarly, if homotypic *Cdc11*–*Cdc11* NC interaction were the sole mode for the polymerization of the majority of the filaments one might expect that a cell expressing *Cdc11*($\Delta\alpha 0$) even in the presence of *Shs1* would be compromised for growth, but that was not so (Figure 3D). Only when the NC contacts in both *Shs1* and *Cdc11* were crippled were cells unable to grow (Figure 3). We feel that these findings provide compelling evidence that, normally, the array of septin structures assembled *in vivo* contains a substantial number of *Shs1*-capped

hetero-octamers interspersed, and associated via an NC interface, with Cdc11-capped hetero-octamers. If so, and given that the length of a septin hetero-octamer is 32 nm (Bertin *et al.* 2008), even the most sophisticated imaging methods available, such as super-resolution fluorescence microscopy (which has a resolution in this same range), may not be adequate to delineate how the components of septin-based structures are arranged. Hence, we continue to pursue EM-based methods for this purpose.

N-terminal polybasic motif is critical for membrane recruitment of septins

Septin structures are tightly apposed to the PM and have been implicated in membrane reorganization, curvature, and even vesicular trafficking (Byers and Goetsch 1976; Tanaka-Takiguchi *et al.* 2009; Saarikangas and Barral 2011; Mostowy and Cossart 2012). Work both *in vitro* (Bertin *et al.* 2010) and *in vivo* (Bridges *et al.* 2014) supports the notion that the presence of membranes promotes filament assembly and the formation of higher-order septin structures. The lipid responsible for high-affinity binding *in vivo* and *in vitro* is the highly anionic species PtdIns4,5P₂ (Zhang *et al.* 1999; Bertin *et al.* 2010). We suspected, therefore, that a correspondingly highly positively charged region in one or more septins might be critical for its recruitment to the membrane and, hence, for its function. The most strikingly basic element in all of the mitotic septins (except Cdc12) is the tract of Lys and Arg associated with $\alpha 0$ (Figure S2). Our mutational analysis (Figure 3) clearly indicated that these basic residues, and not merely the length or α -helical character of this segment of the protein (Figures 3CE, S3), are required for the function of Shs1 and Cdc11, as well as Cdc10 (Figure S2, B and C), which was implicated previously in the association of septin hetero-octamers with lipid monolayers in *in vitro* studies (Bertin *et al.* 2010). Our data further indicate that the basic residues within $\alpha 0$ in Cdc11, Shs1, and Cdc10 are not necessary for formation of either their NC or the G interface (Figure 3, Figure S2, and Figure S3). Moreover, imaging indicated that, for mutants lacking these basic residues, a much higher fraction of them are free in the cytosol. Probably, therefore, one role of $\alpha 0$ in Cdc11 and Shs1 is to present these basic residues, thereby promoting more efficient membrane association as a prelude to the assembly of septin complexes, filaments, and higher-order structures.

The CC element in the CTE mediates the roles of Cdc11 and Shs1 in septin function

Four mitotic septins (*i.e.*, except Cdc10) have a CTE of significant length. Our studies uncovered remarkable distinctions and functional differences among these CTEs. Neither Cdc3 nor Cdc12 tolerated any truncation that significantly changed the distance between the CC elements in their CTEs and the body of their GTP-binding domain. This result is in keeping with other evidence that the CTEs of Cdc3 and Cdc12, which neighbor each other in hetero-octamers, must form a parallel coiled coil. This coiled coil conjoins with the

corresponding Cdc3–Cdc12 coiled coil in a laterally adjacent hetero-octamer. This interaction creates a four-helix bundle that places the side-by-side hetero-octamers in register and seeds formation of paired filaments (Versele *et al.* 2004; Bertin *et al.* 2008, 2010). In marked contrast, the entire spacer separating the CC elements from their G domains could be deleted from the CTEs of Shs1 and Cdc11 without loss of function (Figure S2 and Figure 5). Moreover, the CTE domains of Shs1 and Cdc11 could be swapped, as long as they were located on the two terminal subunits, but not when placed on two other immediately juxtaposed, but internally located, subunits (Cdc10) (Figure 6). Thus, the function of the CTEs of the terminal subunits is not spatially restricted within filament superstructure as long as they are positioned at the junction between neighboring octamers. These observations provide additional support for the conclusion that *in vivo* Shs1 is located in close apposition to Cdc11 and not in distinct or separate ensembles (Figure 7).

Despite the fact that Cdc11 and Shs1, as the terminal subunits of hetero-octamers, contribute to both the assembly and supramolecular architecture of septin-based structures (Bertin *et al.* 2008; Garcia *et al.* 2011; McMurray *et al.* 2011), we found that the CTE domains of Cdc11 and Shs1 are not required for their bud neck localization (Figure 4), which requires hetero-octamer assembly and filament formation. Yet, cells in which the CTEs of both Cdc11 and Shs1 are missing are inviable (Figure 4). Therefore, it seems likely that the CC elements in the CTEs of Shs1 and Cdc11 share the function of interacting with and thereby recruiting nonseptin proteins that must execute their action in association with the septin structures at the bud neck. In this regard, a recent study reported that the CTE of Shs1 interacts with an ER protein (Scs2) and aids in creation of a barrier that prevent diffusion of the ER between a mother and daughter cell (Chao *et al.* 2014). However, genetic or physical links between Shs1 (and/or Cdc11) and other cellular proteins involved in morphogenesis at the bud neck have also been described, including IQGAP Iqg1 (Iwase *et al.* 2007), formin Bnr1 (Buttery *et al.* 2012), F-BAR protein Hof1 (Meitinger *et al.* 2013), polarisome component Spa2 (Mino *et al.* 1998), septin-interacting protein Nis1 (Iwase and Toh-E 2001), and the Myo1-binding factor Bni5 (Lee *et al.* 2002). As described in detail in the accompanying article (Finnigan *et al.* 2015), in our hands, the critical function shared by the CC elements in the CTEs of Shs1 and Cdc11 is physical association with Bni5.

Coevolution of paralogous septin subunits provides diversification and additional complexity in septin structure and function

After it was shown that Shs1 could occupy the same terminal position as Cdc11 in yeast septin hetero-octamers and confer different properties on the resulting complexes (Garcia *et al.* 2011), it became apparent that, for mammalian septin complexes, placement of alternative subunits at different positions within hetero-octamers would likely provide a mechanism to impart differential features or functions appropriate

to the septin structures in particular cell types or at particular stages in development (Saarikangas and Barral 2011; Mostowy and Cossart 2012; Sellin *et al.* 2012; Fung *et al.* 2014; Sellin *et al.* 2014). Therefore, understanding how paralogous subunits, like *Cdc11* and *Shs1*, have coevolved within the same complex and in the same cell type is of significant interest. Superficially, duplication of an ancestral gene eventually gave rise to one essential subunit (*Cdc11*) and one “dispensable” subunit (*Shs1*). It is telling, however, that over evolutionary time the *SHS1* gene was neither lost nor pseudogenized, but retained (Pan *et al.* 2007). In this regard, we have found that the *Shs1* ortholog from the filamentous fungus *A. gossypii* [as well as the *Shs1* orthologs from both *C. glabrata* and *C. albicans* (our unpublished results)] is able to associate with *S. cerevisiae* *Cdc12*, as judged by two independent criteria. First, like ScShs1, expression of AgShs1 effectively caps *Cdc12*–*Cdc3*–*Cdc10*–*Cdc10*–*Cdc3*–*Cdc12* heterohexamers and prevents the growth of *cdc11Δ* *S. cerevisiae* cells (Figure S6A). Second, fluorescently tagged AgShs1 localizes exclusively to the bud neck (Figure S6B), as seen previously by others (Meseroll *et al.* 2012). Yet, we showed clearly that AgShs1 is unable to functionally replace its *S. cerevisiae* counterpart (Figure S6A). Thus, our findings raise an important but underappreciated caveat—in the absence of independent tests for functionality, incorporation at the bud neck alone cannot be equated with retention of biological function.

We traced the incompatibility of AgShs1 in complementing loss of ScShs1 to some feature of its globular G domain, whereas its CTE, in which the CC element is the only portion of the sequence highly conserved between *A. gossypii* and *S. cerevisiae* (Figure S6C), had the capacity to function in budding yeast. These findings provide additional support for the conclusion that the CC segment of *Shs1* is the sequence feature in its CTE most critical for the physiological action of this subunit. Moreover, although we found a partially overlapping role for the CC element in the CTE of *Cdc11*, our data also show that the CTEs of *Shs1* and *Cdc11* have each evolved a unique function(s) that cannot be replaced by the other (Figure 6). Furthermore, *Cdc11* and *Shs1* clearly compete for occupancy of the terminal position in hetero-octamers because occupancy of that site by one is readily perturbed by overexpression of the other (our unpublished results). It is likely, therefore, that during their divergence, *CDC11* and *SHS1* would have undergone selection for the appropriate degree of modulation of their relative levels of expression. Finally, the fact that both *Cdc11* and *Shs1* retained the capacity to bind to *Cdc12* indicates that a G interface can be rather flexible, accommodating different interaction partners even across large spans of evolutionary time. Indeed, just as budding yeast *Cdc12* is able to bind either *Cdc11* or *Shs1* (Garcia *et al.* 2011), the equivalent human subunit (SEPT7) is able to bind any of three alternative terminal subunits (SEPT3, SEPT9, or SEPT12), each of which is produced in a variety of splice and translational variants (Roeseler *et al.* 2009; Hall and Russell 2012; Fung *et al.* 2014).

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Supporting Information

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Comprehensive Genetic Analysis of Paralogous Terminal Septin Subunits Shs1 and Cdc11 in *Saccharomyces cerevisiae*

Gregory C. Finnigan, Julie Takagi, Christina Cho, and Jeremy Thorner

SUPPORTING INFORMATION

for

Comprehensive genetic analysis of paralogous terminal septin subunits

Shs1 and Cdc11 in *Saccharomyces cerevisiae*

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Figure S1. Further analysis of Shs1 and Cdc11 alleles. (A) BY4741, GFY-568, GFY-58 and GFY-153 were grown overnight in SD-Ura at 30°C and spotted onto plates without or with 5-FOA. BY4741 (WT) contained empty vector (pRS316). In *CDC11**, the *CDC11* ORF is immediately followed by the *ADH1*(term)::Hyg^R cassette. *CDC11-mC* is Cdc11-mCherry. (B) GFY-140, GFY-87 and GFY-137 were grown overnight in YPGal at 25°C and spotted on Gal-containing plates without or with 5-FOA to select against the *URA3*-marked *CDC10*-expressing plasmid (present in all strains). (C) Test of endogenously-expressed Shs1(S221A)-eGFP and Shs1(S221D)-eGFP in three conditions where Shs1 is required for growth (*cdc10Δ*, *cdc11-CTEΔ-mCherry* and *cdc12-6*) in GFY-87, GFY-137, GFY-310 and GFY-188 as in (B) and in GFY-293, GFY-166, GFY-292, GFY-294, GFY-302, GFY-139, GFY-297, GFY-298, GFY-160, GFY-147, GFY-163, GFY-923 and GFY-951 as in (A), except those carrying *cdc12-6* were incubated at 25°C, and one condition where Shs1 inhibits growth (MCMURRAY *et al.* 2011). (D) *Upper*, region of Shs1 containing Ser221 (red triangle) aligned with Cdc11 using CLUSTAL-W (THOMPSON *et al.* 1994); +1 Phe is preferred phosphoacceptor motif for protein kinase Rad53 kinase (MOK *et al.* 2010). *Lower*, GFY-188 and GFY-310 from (C, *top*) were transformed with plasmid (pGF-preIVL6) expressing Cdc10-mCherry, selected on 5-FOA to remove the *URA3*-marked plasmid expressing untagged Cdc10, grown to exponential phase, and viewed by fluorescence microscopy. Dotted line, cell periphery. (E) To test functionality of the indicated *SHS1* alleles in *hof1Δ* cells, YFR-387, GFY-934, GFY-971, GFY-978 and GFY-966 were grown overnight at 30°C and spotted as in (B); covering plasmid expressing *HOF1* was pJT4836.

A

		$\alpha 0$ helix		P-loop	
HsSEPT2	MSKQQPTQFINPETPGYVGF	ANLPNQV HRKSVKK	GFEFTLMVV	GESGLGKS	61
ScCdc12	MSAATATAAPVPPPVGI	SNLPNQ RYK IVNEE	GGTFTVMLC	GESGLGKT	48
ScCdc10	MDPLSSVQPASYVGF	DTITNQIE HRLLKK	GFQFNIMVV	GQSGLGKS	46
ScCdc3	MSL (80) PDQPEIKFIRQINGYVGF	ANLP KQW HRRSIKN	GFSFNLLCV	GPDGIGKT	133
ScCdc11	MSGII	DASSAL RKRKHLKR	GITFTVMIV	GQSGSGRS	36
ScShs1	MSTAST	PPINLF RRKKEHKR	GITYTMLLC	GPAGTGKT	37

B

C

Figure S2. Mutational analysis of the poly-basic motif in the N-terminal $\alpha 0$ helix in Cdc10. (A) Based on sequence homology and modeling (MCMURRAY AND THORNER 2008) against the crystal structures of human septins (SIRAJUDDIN *et al.* 2007), the residues in the $\alpha 0$ helix in human SEPT2 (purple frame) are aligned to the corresponding residues in the indicated yeast septins. Basic residues, red; residues corresponding to the P-loop diagnostic of all Ras-related GTP-binding proteins (SPRANG 1997; WITTINGHOFFER AND VETTER 2011), green frame. (B) GFY-42 and GFY-395 expressing either *CDC10-mCherry* or *cdc10(H24A R25A K28A K29A)-mCherry* ("*cdc10-NT-A-mC*") from the endogenous *CDC10* locus were selected twice on 5-FOA at room temperature to remove the covering plasmid expressing *CDC10*, grown overnight at 25°C in YPD, spotted onto the same medium, and incubated at the indicated temperatures. (C) *Left* (fluorescence image), GFY-42 and GFY-395 expressing either *CDC10-mCherry* or *cdc10(H24A R25A K28A K29A)-mCherry* ("*cdc10-NT-A-mC*") from the endogenous *CDC10* locus carrying the covering plasmid expressing *CDC10*. Dotted line, cell periphery. *Right* (DIC image), same strains after selection at 25°C for loss of the covering plasmid expressing *CDC10*, grown overnight at 25°C in YPD, back-diluted, and then incubated at 30°C for 4.5 hours prior to viewing. Four independent fields are shown for each strain. Black triangles, cells with markedly elongated buds and/or abnormal mother cell morphology.

A **B**

Figure S3. Basic residues are a critical functional element of the N-terminal $\alpha 0$ helix in both Shs1 and Cdc11. (A) Functionality of four endogenously-expressed *SHS1* mutants (each tagged with C-terminal eGFP)— a null allele (*shs1Δ*), a deletion of $\alpha 0$ [*shs1(Δ2-18)*], Ala substitution of all basic residues in $\alpha 0$ [*shs1(R13A R14A K15A K16A K19A R20A)* ("*shs1-NT-A*")], and the $\alpha 0$ deletion combined with Ala substitution of the remaining adjacent basic residues [*shs1(Δ2-18; K19A R20A)*] —were tested in one of the Shs1-dependent genetic backgrounds described in this study by growing the corresponding strains (GFY-87, GFY-137, GFY-93, GFY-249 and GFY-1021) overnight in YPGal at 25°C and spotting onto Gal medium in the absence or presence of 5-FOA (to select against the covering *CDC10*-expressing plasmid). (B) As indicated, combinations of an $\alpha 0$ deletion (residues 2-18Δ for both Cdc11 and Shs1) without and with Ala substitution mutation of the remaining adjacent basic residue(s), R19 in Cdc11 and K19 and R20 in Shs1 (see Fig. S2A), were tested for function by growing the corresponding strains (GFY-58, GFY-164, GFY-1024, GFY-1062, GFY-1061, GFY-147 and GFY-1022) overnight in SD-Ura medium at 30°C and then spotting onto medium in the absence or presence of 5-FOA (to select against the covering *CDC11*-expressing plasmid). The *SHS1* derivatives in these strains contained a C-terminal eGFP tag.

Figure S5. Presence of a C-terminal mCherry tag or G domain mutation G29D reduces Cdc11 function providing additional sensitized genetic backgrounds to assess Shs1 functionality. (A) GFY-160, GFY-161 and GFY-162 were grown overnight in SD-Ura at 30°C and spotted onto medium without or with 5-FOA (to remove the covering *CDC11*-expressing plasmid). Each Cdc11 construct contained a C-terminal mCherry tag and each Shs1 construct contained a C-terminal eGFP tag. (B) Strains (GFY-532, GFY-533 and GFY-534) otherwise identical to those in (A) were constructed in which the mCherry tag was replaced with the *ADH1(term)::Hyg^R* cassette inserted immediately after the *CDC11* ORF (indicated by an asterisk) and first propagated on 5-FOA medium (to select against the covering *CDC11*-expressing plasmid), then grown overnight in rich medium at 25°C, and finally spotted on plates and incubated at either 25°C or 37°C, as indicated. (C) Derivatives (GFY-724, GFY-725 and GFY-726) lacking the *SHS1* gene were generated from the same strains in (B) and then were tested as in (A). (D) Strains (GFY-681, GFY-694 and GFY-675) in which the *CDC11* alleles shown in (B) also harbored a G29D mutation (*cdc11-7*) within the GTP-binding domain (NAGARAJ *et al.* 2008; WEEMS *et al.* 2014), and in which the *SHS1* locus lacked any fluorescent tag, were grown and spotted as in (A).

Figure S6. *A. gossypii* Shs1 localizes to the bud-neck, but does not functionally replace *S. cerevisiae* Shs1. (A) Functionality of intact Ag Shs1 and two chimeric protein derivatives (one in which the CTE of Ag Shs1 was replaced with the CTE of Sc Shs1, and the other in which the CTE of Sc Shs1 was replaced with the CTE of Ag Shs1), each tagged at its C-terminus with eGFP and expressed from the endogenous Sc *SHS1* promoter, were tested in two Shs1-dependent genetic backgrounds described in this study, *cdc10Δ* (upper panel) and *cdc11-CTEΔ-mCherry* (middle panel). For the former, overnight cultures of GFY-87, GFY-137, GFY-94, GFY-644, GFY-655 and GFY-643 were grown in YPGal at 25°C before spotting onto Gal medium without and with 5-FOA (to select against the covering *URA3*-marked *CDC10*-expressing plasmid). For the latter, GFY-160, GFY-162, GFY-615, GFY-571 and GFY-614 were grown overnight in SD-Ura at 30°C before spotting onto medium without and with 5-FOA (to select against the covering *URA3*-marked *CDC11*-expressing plasmid). Lower panel, expression of *SHS1* in cells lacking Cdc11 is lethal (because Shs1 caps the ends of Cdc12-Cdc3-Cdc10-Cdc10-Cdc3-Cdc12 hexamers) (MCMURRAY *et al.* 2011). Likewise, each of the Ag Shs1 constructs was unable to support the growth of *cdc11Δ* cells, indicating that all three were able to form a G-interface with Cdc12. For this test, GFY-160, GFY-147, GFY-149, GFY-639, GFY-637 and GFY-683 were grown and spotted as for the middle panel. (B) GFY-643, GFY-644 and GFY-655 were transformed with a vector expressing *CDC10-mCherry* (pGF-preIVL6), selected twice on medium containing 5-FOA to remove the covering *URA3*-marked plasmid expressing WT *CDC10*-expressing vector, grown to mid-exponentially phase in SD-Leu medium, and viewed by fluorescence microscopy. Cell perimeter, white dotted lines. (C) Alignment of the CTEs of Ag Shs1 and Sc Shs1, as in Fig. S4A, with the position chosen as the breakpoint for the CTE domain swaps indicated by the red triangle.

Table S1. Yeast strains used to obtain the data shown in the Figures S1 to S6.

Strain	Genotype	Reference
GFY-140	BY4741; <i>cdc10Δ::Hyg^R</i> + <i>pJT2022</i>	This study
GFY-87	BY4741; <i>cdc10Δ::Kan^R shs1Δ::SHS1::eGFP::Nat^R</i> + <i>pJT2022</i>	This study
GFY-137	BY4741; <i>cdc10Δ::Kan^R shs1Δ::Hyg^R</i> + <i>pJT2022</i>	This study
BY4741	<i>MATa leu2Δ ura3Δ met15Δ his3Δ</i>	(BRACHMANN <i>et al.</i> 1998)
GFY-568	BY4741; <i>cdc11Δ::CDC11::Hyg^R</i> + <i>pJT1520</i>	This study
GFY-58	BY4741; <i>cdc11Δ::CDC11::mCherry::SpHIS5</i> + <i>pJT1520</i>	This study
GFY-153	BY4741; <i>cdc11Δ::Kan^R</i> + <i>pJT1520</i>	This study
GFY-310	BY4741; <i>cdc10Δ::Kan^R shs1Δ::shs1(S221D)::eGFP::Nat^R</i> + <i>pJT2022</i>	This study
GFY-188	BY4741; <i>cdc10Δ::Kan^R shs1Δ::shs1(S221A)::eGFP::Nat^R</i> + <i>pJT2022</i>	This study
GFY-293	BY4741; <i>cdc11Δ::cdc11(Δ357-415)::mCherry::SpHIS5 shs1Δ::SHS1::eGFP::Nat^R</i> + <i>pJT1520</i>	This study
GFY-166	BY4741; <i>cdc11Δ::cdc11(Δ357-415)::mCherry::SpHIS5 shs1Δ::Hyg^R</i> + <i>pJT1520</i>	This study
GFY-292	BY4741; <i>cdc11Δ::cdc11(Δ357-415)::mCherry::SpHIS5 shs1Δ::shs1(S221D)::eGFP::Nat^R</i> + <i>pJT1520</i>	This study
GFY-294	BY4741; <i>cdc11Δ::cdc11(Δ357-415)::mCherry::SpHIS5 shs1Δ::shs1(S221A)::eGFP::Nat^R</i> + <i>pJT1520</i>	This study
GFY-302	BY4741; <i>cdc12Δ::cdc12(K391N Δ392-407)::Hyg^R shs1Δ::SHS1::eGFP::Nat^R</i> + <i>pJT1622</i>	This study

GFY-139	BY4741; <i>cdc12Δ::cdc12(K391N Δ392-407)::Hyg^R</i> <i>shs1Δ::Kan^R + pJT1622</i>	This study
GFY-297 ⁱ	BY4741; <i>cdc12Δ::cdc12(K391N Δ392-407)::Hyg^R</i> <i>shs1Δ::shs1(S221D)::eGFP::Nat^R + pJT1622</i>	This study
GFY-298	BY4741; <i>cdc12Δ::cdc12(K391N Δ392-407)::Hyg^R</i> <i>shs1Δ::shs1(S221A)::eGFP::Nat^R + pJT1622</i>	This study
GFY-160	BY4741; <i>cdc11Δ::CDC11::mCherry::SpHIS5^R</i> <i>shs1Δ::SHS1::eGFP::Nat^R + pJT1520</i>	This study
GFY-147	BY4741; <i>cdc11Δ::Kan^R shs1Δ::SHS1::eGFP::Nat^R +</i> <i>pJT1520</i>	This study
GFY-163	BY4741; <i>cdc11Δ::Kan^R shs1Δ::Hyg^R + pJT1520</i>	This study
GFY-923	BY4741; <i>cdc11Δ::Kan^R shs1Δ::shs1(S221A)::eGFP::Nat^R +</i> <i>pJT1520</i>	This study
GFY-951	BY4741; <i>cdc11Δ::Kan^R shs1Δ::shs1(S221D)::eGFP::Nat^R +</i> <i>pJT1520</i>	This study
YFR-387	BY4742; <i>hof1Δ::Kan^R + pJT4836</i>	This study
GFY-934	BY4742; <i>hof1Δ::Kan^R shs1Δ::Hyg^R + pJT4836</i>	This study
GFY-971	BY4741; <i>hof1Δ::Kan^R shs1Δ::SHS1::eGFP::Nat^R + pJT4836</i>	This study
GFY-978	BY4741; <i>hof1Δ::Kan^R shs1Δ::shs1(Δ349-551)::eGFP::Nat^R +</i> <i>pJT4836</i>	This study
GFY-966	BY4741; <i>hof1Δ::Kan^R shs1Δ::shs1(S350A T351A T386A</i> <i>S416A S441A S447A T454A S460A T462A S519A S520A</i> <i>S521A S522A S525A S529A S530A T539A T541A</i> <i>S545A)::eGFP::Nat^R + pJT4836</i>	This study
GFY-42	BY4741; <i>cdc10Δ::CDC10::mCherry::SpHIS5</i>	This study

GFY-395	BY4741; <i>cdc10Δ::cdc10(H24A R25A K28A K29A)::mCherry::SpHIS5</i>	This study
GFY-164	BY4741; <i>cdc11Δ::CDC11::mCherry::SpHIS5^R shs1Δ::Hyg^R + pJT1520</i>	This study
GFY-1024	BY4741; <i>cdc11Δ::cdc11(Δ2-18 R19A)::mCherry::SpHIS5 + pJT1520</i>	This study
GFY-1062	BY4741; <i>cdc11Δ::cdc11(Δ2-18 R19A)::mCherry::SpHIS5 shs1Δ::Hyg^R + pJT1520</i>	This study
GFY-1061	BY4741; <i>cdc11Δ::cdc11(Δ2-18 R19A)::mCherry::SpHIS5 shs1Δ::shs1(Δ2-18Δ K19A R20A)::eGFP::Nat^R + pJT1520</i>	This study
GFY-1022	BY4741; <i>cdc11Δ::Kan^R shs1Δ::shs1(Δ2-18 K19A R20A)::eGFP::Nat^R + pJT1520</i>	This study
GFY-93	BY4741; <i>cdc10Δ::Kan^R shs1Δ::shs1(Δ2-18)::eGFP::Nat^R + pJT2022</i>	This study
GFY-249	BY4741; <i>cdc10Δ::Kan^R shs1Δ::shs1(R13A R14A K15A K16A K19A R20A)::eGFP::Nat^R + pJT2022</i>	This study
GFY-1021	BY4741; <i>cdc10Δ::Kan^R shs1Δ::shs1(Δ2-18Δ K19A R20A)::eGFP::Nat^R + pJT2022</i>	This study
GFY-161	BY4741; <i>cdc11Δ::cdc11(Δ2-1Δ)::mCherry::SpHIS5^R shs1Δ::shs1(Δ2-18)::eGFP::Nat^R + pJT1520</i>	This study
GFY-162	BY4741; <i>cdc11Δ::cdc11(Δ357-415)::mCherry::SpHIS5 shs1Δ::shs1(Δ349-551)::eGFP::Nat^R + pJT1520</i>	This study
GFY-532	BY4741; <i>cdc11Δ::CDC11::Hyg^R shs1Δ::SHS1::eGFP::Nat^R + pJT1520</i>	This study
GFY-533	BY4741; <i>cdc11Δ::cdc11(Δ2-18)::Hyg^R shs1Δ::shs1(Δ2-</i>	This study

	18)::eGFP::Nat ^R + pJT1520	
GFY-534	BY4741; <i>cdc11Δ::cdc11(Δ357-415)::Hyg^R</i> <i>shs1Δ::shs1(Δ349-551)::eGFP::Nat^R + pJT1520</i>	This study
GFY-724	BY4741; <i>cdc11Δ::CDC11::Hyg^R shs1Δ::Kan^R + pJT1520</i>	This study
GFY-725	BY4741; <i>cdc11Δ::cdc11(Δ2-18)::Hyg^R shs1Δ::Kan^R +</i> <i>pJT1520</i>	This study
GFY-726	BY4741; <i>cdc11Δ::cdc11(Δ357-415)::Hyg^R shs1Δ::Kan^R +</i> <i>pJT1520</i>	This study
GFY-681	BY4741; <i>cdc11Δ::cdc11(G29D)::SpHIS5 + pJT1520</i>	This study
GFY-694	BY4741; <i>cdc11Δ::cdc11(Δ2-18 G29D)::SpHIS5 + pJT1520</i>	This study
GFY-675	BY4741; <i>cdc11Δ::cdc11(G29D Δ357-415)::SpHIS5 +</i> <i>pJT1520</i>	This study
GFY-94	BY4741; <i>cdc10Δ::Kan^R shs1Δ::shs1(Δ349-551)::eGFP::Nat^R</i> <i>+ pJT2022</i>	This study
GFY-644	BY4741; <i>cdc10Δ::Kan^R shs1Δ::Sc_shs1(1-</i> <i>339)::Ag_shs1(336-580)::eGFP::Nat^R + pJT2022</i>	This study
GFY-655	BY4741; <i>cdc10Δ::Kan^R shs1Δ::Ag_shs1(1-</i> <i>335)::Sc_shs1(340-551)::eGFP::Nat^R + pJT2022</i>	This study
GFY-643	BY4741; <i>cdc10Δ::Kan^R shs1Δ::Ag_shs1(1-580)::eGFP::Nat^R</i> <i>+ pJT2022</i>	This study
GFY-615	BY4741; <i>cdc11Δ::cdc11(Δ357-415)::mCherry::SpHIS5</i> <i>shs1Δ::Sc_shs1(1-339)::Ag_shs1(336-580)::eGFP::Nat^R +</i> <i>pJT1520</i>	This study
GFY-571	BY4741; <i>cdc11Δ::cdc11(Δ357-415)::mCherry::SpHIS5</i> <i>shs1Δ::Ag_shs1(1-335)::Sc_shs1(340-551)::eGFP::Nat^R +</i>	This study

pJT1520

GFY-614	BY4741; <i>cdc11Δ::cdc11(Δ357-415)::mCherry::SpHIS5</i> <i>shs1Δ::Ag_shs1(1-580)::eGFP::Nat^R</i> + <i>pJT1520</i>	This study
GFY-149	BY4741; <i>cdc11Δ::Kan^R shs1Δ::shs1(Δ349-551)::eGFP::Nat^R</i> + <i>pJT1520</i>	This study
GFY-639	BY4741; <i>cdc11Δ::Kan^R shs1Δ::Sc_shs1(1-339)::Ag_shs1(336-580)::eGFP::Nat^R</i> + <i>pJT1520</i>	This study
GFY-637	BY4741; <i>cdc11Δ::Kan^R shs1Δ::Ag_shs1(1-335)::Sc_shs1(340-551)::eGFP::Nat^R</i> + <i>pJT1520</i>	This study
GFY-683	BY4741; <i>cdc11Δ::Kan^R shs1Δ::Ag_shs1(1-580)::eGFP::Nat^R</i> + <i>pJT1520</i>	This study

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