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Native cysteine residues are dispensable for the structure and function of all five yeast mitotic septins

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Abstract

Budding yeast septins assemble into hetero-octamers and filaments required for cytokinesis. Solvent-exposed cysteine (Cys) residues provide sites for attaching substituents useful in assessing assembly kinetics and protein interactions. To introduce Cys at defined locations, site-directed mutagenesis was used, first, to replace the native Cys residues in Cdc3 (C124 C253 C279), Cdc10 (C266), Cdc11 (C43 C137 C138), Cdc12 (C40 C278), and Shs1 (C29 C148) with Ala, Ser, Val, or Phe. When plasmid-expressed, each Cys-less septin mutant rescued the cytokinesis defects caused by absence of the corresponding chromosomal gene. When integrated and expressed from its endogenous promoter, the same mutants were fully functional, except Cys-less Cdc12 mutants (which were viable, but exhibited slow growth and aberrant morphology) and Cdc3(C124V C253V C279V) (which was inviable). No adverse phenotypes were observed when certain pairs of Cys-less septins were co-expressed as the sole source of these proteins. Cells grew less well when three Cys-less septins were co-expressed, suggesting some reduction in fitness. Nonetheless, cells chromosomally expressing Cys-less Cdc10, Cdc11, and Cdc12, and expressing Cys-less Cdc3 from a plasmid, grew well at 30ºC. Moreover, recombinant Cys-less septins—or where one of the Cys-less septins contained a single Cys introduced at a new site—displayed assembly properties *in vitro* indistinguishable from wild-type.

Keywords

Saccharomyces cerevisiae; site-directed mutagenesis; genetic complementation; gene replacement; protein purification; electron microscopy

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INTRODUCTION

Septins are a class of eukaryotic GTP-binding proteins.[1,2] They localize to sites of cytokinesis and are essential for cell division in budding yeast (*Saccharomyces cerevisiae*), *Drosophila* embryos, and cultured mammalian cells.[3–5] In mitotic yeast, five septins (Cdc3, Cdc10, Cdc11, Cdc12, and Shs1) are expressed. Cytokinesis in yeast occurs at the neck between a mother cell and its bud; septin-based structures line the cell cortex at this location and are readily visualized using any septin tagged with a fluorophore.[6] Whether purified from yeast[7] or as recombinant proteins from *E. coli*,[8] yeast septins associate into hetero-octameric complexes. Electron microscopy (EM) revealed that the complexes are linear rods.[9] Rods with the order Cdc11–Cdc12–Cdc3–Cdc10–Cdc10–Cdc3–Cdc12–Cdc11 polymerize end-on-end at low salt into straight paired filaments.[9] Rods with an alternate terminal subunit (Shs1–Cdc12–Cdc3–Cdc10–Cdc10–Cdc3–Cdc12–Shs1) assemble into spirals and rings.[10] Both structures are reminiscent of septin organization at the bud neck *in vivo*, visualized using EM techniques (thin-section, rapid-freeze and deep-etch, and thick-section tomography).[11–13] The supramolecular architecture of the collar of septins at the bud neck is most compatible with a stack of interconnected circumferential rings or a helical network of filaments closely associated with the plasma membrane.[11,14] These structures disappear at the restrictive temperature in cells carrying temperature-sensitive septin mutations.[15–17]

During bud growth, the septin collar acts as a scaffold to recruit an actomyosin contractile ring, the apparatus that drives plasma membrane ingression during septation,[5,6] and regulatory factors that upon septin assembly, license the G2/M transition.[18,19] Many proteins localize to the bud neck in a septin-dependent manner, but direct physical interaction with a septin been demonstrated for only a few.[14,20] Thus, mechanistic details of the scaffold function of septins are still unclear. At cytokinesis, the septin collar splits, leaving two rings that act as diffusion barriers, restricting movement of neck-localized factors (e.g., exocyst complex, which directs localized exocytosis) and cell wall remodeling enzymes (e.g., Chs2, the chitin synthase that deposits the primary septum).[21,22] Septin complexes unable to form filaments do not stably localize to the bud neck,[23] precluding the above cytokinetic functions, thereby explaining why sep-tin filament formation is essential for cell division. How transitions in septin organization occur during the cell cycle and are linked to cell cycle progression remain largely unexplored, although post-translational modifications and interactions with polarity proteins likely play important roles.[5,6,10,24]

Factors controlling the dynamics of septin organization *in vivo* are likely to affect the assembly kinetics of septin structures *in vitro*. Prior studies of filament formation by septin complexes have relied on cumbersome EM or sedimentation methods.[8,9,25] Real-time assays for polymerization have provided substantial mechanistic insight into the assembly properties and regulation of other cytoskeletal polymers. For tubulin,[26] increased light scattering provides a read-out of polymerization, and has been used to assess septin filament formation.[54] However, for septins, this approach could be misleading, given their propensity for non-native associations[23,27] (including homotypic beta-amyloid-like aggregation[28,29]). In this regard, actin filament assembly provides an instructive example; a thiol-reactive derivative of pyrene can be attached to a single solvent-exposed Cys and emission of this fluorescent probe undergoes a marked change upon actin polymerization.[30] A variation on this tactic is more applicable to septins; a reactive Cys in ParM[31] or MreB[32] (bacterial actin homologs) was labeled with either of two fluorescent dyes whose excitation and emission properties allow Förster Resonance Energy Transfer (FRET) upon mixing of the differentially tagged proteins under polymerization conditions. In theory, this same approach should permit convenient monitoring of the formation of the Cdc11-Cdc11 interface that mediates authentic septin filament assembly. Additionally, bifunctional or photoactivable

crosslinkers can be attached to solvent-exposed Cys to couple (or transfer a label) to an interacting protein.[33,34] This strategy would allow unbiased identification of cellular proteins that intimately associate with septin filaments.

To prepare septin structures that harbor lone surface-exposed Cys, it is first necessary to replace endogenous Cys while preserving septin function. As shown here, all endogenous Cys in each of the five mitotic septins were substituted with a near-isosteric counterpart (Ala or Ser) or with another option (Val or Phe) that phylogenetic comparisons revealed is a residue frequently found at the corresponding position in related septins. Each construct was tested for retention of biological function by genetic complementation. We further examined what combinations of co-expressed Cys-less septins were tolerated by cells. Finally, we expressed recombinantly Cdc3, Cdc10, Cdc11, and Cdc12 variants with no Cys (or with a single Cys residue introduced at a new location in each one of these otherwise Cys-less septins) and found that all were incorporated into hetero-octameric complexes that were capable of assembling into filaments *in vitro*. These studies establish a potentially useful system for study of septin filament assembly and function.

MATERIALS AND METHODS

Strains and growth conditions

Yeast strains (Table I) were cultivated on rich (YPD) or defined (SC) medium[35] supplemented appropriately to select for plasmids or integrants. Analog 5-fluoro-orotate (5-FOA; Toronto Research Chemicals) was used for *URA3* counter-selection.[36] *E. coli* DH5α was used for plasmid construction, and BL21(DE3) for IPTG-induced expression of T7-promoter-driven recombinant septins.

Plasmids and recombinant DNA methods

Plasmids (Table II) were constructed via site-directed Cys mutagenesis using a two-stage PCR protocol[37] with Pfu and Pfu Turbo polymerases (Stratagene), and confirmed by sequencing (primers listed in Table III). In some cases, two sets of primers were used in the PCR reaction to introduce multiple mutations from the same template. Linear DNA for recombination-mediated chromosomal integration was produced using Phusion High-Fidelity DNA polymerase (Finnzymes) from centromeric plasmid templates, and included the appropriate portion of the septin ORF and a selectable marker located immediately downstream, the only exceptions were *CDC12* integration products, which were constructed using an overlap PCR technique to introduce a downstream *LEU2* marker (see Table I). After amplification, digestion with *Dpn*I (NEB) ensured destruction of the Dam-methylated templates.[37] Yeast transformation was conducted by standard methods.[38] Correct integration was confirmed by DNA sequencing of PCR products amplified from genomic DNA extracted from transformants.[39] Some combinations of septin variants were obtained by mating haploid strains, inducing meiosis and sporulation in the resulting diploid cells, and isolating the desired spore progeny by random spore analysis.[40]

Preparation of yeast cell extracts and immunoblotting

Yeast cells were lysed using a post-alkali treatment SDS-boiling method.[41] Samples (30 μg total protein) were resolved by SDS-PAGE, blotted onto nitrocellulose filters, exposed to a primary anti-septin IgG, followed by an appropriate secondary anti-IgG antibody tagged with an infrared fluorescent dye (Rockland Immunochemicals or Molecular Probes/Invitrogen), and imaged using an Odyssey™ infrared imager (Li-Cor Biosciences). Anti-septin IgGs were: anti-Cdc3;[42] anti-Cdc10 (Santa Cruz); anti-Cdc11 (Santa Cruz); anti-Cdc12;[43] and anti-Shs1.[44]

Fluorescence microscopy

Cells from cultures in mid-exponential phase were transferred to 2% agarose pads on glass slides and viewed immediately under the 100X objective of a BH-2 epifluorescence microscope (Olympus) equipped with a GFP band-pass filter (Chroma). Images were collected with a charged-coupled device camera (Olympus) and processed with Magnafire SP imaging software (Optronics) and processed using Photoshop (Adobe).

Preparation of recombinant septin complexes, in vitro biotinylation of Lys or Cys, and EM analysis

Cdc3, Cdc10, Cdc11, and $(His)_6$-tagged Cdc12 (and/or Cys-less variants) were co-expressed in *E. coli* and the resulting complexes were purified using three chromatographic steps.[9] EZ-Link™-Sulfo-NHS-LC-LC-biotin and Mts-Atf-Biotin (Pierce/Thermo) were dissolved in phosphate-buffered saline and added to purified complexes, following the manufacturer's recommendations. For EM visualization, complexes were diluted to 0.01 mg/mL, adsorbed onto carbon-coated grids, stained with 2% uranyl formate, and examined using a Tecnai T12 microscope (FEI) operated at 120 kV. Images were collected using a Gatan 1024X1024 CCD camera at 30,000× with a 1 μm underfocus.

RESULTS AND DISCUSSION

Solvent accessibility of endogenous Cys residues in yeast septins

To assess solvent accessibility of endogenous Cys, purified septin hetero-octamers were incubated with a thiol-reactive biotin-containing probe. The resulting products were resolved by SDS-PAGE and incubated with fluorescently labeled streptavidin [Fig. 1(A)]. As a control for both specificity and relative labeling efficiency, an amino-reactive biotin-containing probe was used to modify Lys [Cdc3 (55), Cdc10 (16), Cdc11 (20) and Cdc12 (30)]. The intensity of Cys labeling of each septin was roughly commensurate with the total Cys in each protein [Cdc3 (3), Cdc10 (1), Cdc11 (3), and Cdc12 (2)], except for Cdc11. Modeling of the approximate positions of these Cys [Fig. 1(B)] based on crystal structures of septins in a human hetero-oligomer[45,46] suggests that the tandem pair (C137 C138) in Cdc11 [Fig. 1(C)] may be buried and only C43 accessible for labeling.

Given their solvent accessibility, we systematically eliminated all endogenous Cys to avoid any reactivity. We used a phylogenetic approach to select appropriate substitution mutations that would best preserve native fold and function. Septin family members share ≥30% sequence identity and even higher similarity [Fig. 1(C)]. Based on sequence alignments, we chose non-Cys residues found at equivalent positions in closely related sep-tins. Hence, C266 in Cdc10 and C278 in Cdc12 were changed to Ser as found in the homeologous septins Cdc3 and Cdc11 [Fig. 1(C)]. Following the same logic, certain Cys were substituted with Ala (e.g., C137 in Cdc11). We also considered non-Cys residues found in septins from other species, especially human Sept2, Sept6, and Sept7, where crystal structures are available.[45–48] Thus, C124 in Cdc3, C40 in Cdc12, and C29 in Shs1 were mutated to Val because Val frequently appears at the equivalent positions in other yeast septins and/or in one or more human septin [Fig. 1(C)]. For the same reason, C43 in Cdc11 was mutated to Phe [Fig. 1(C)], despite the fact that Phe is more bulky. In the human Sept7–Sept6–Sept2–Sept2–Sept6–Sept7 hetero-oligomer,[45,46] Phe at this position is situated within the Sept6-Sept7 and Sept2-Sept2 interfaces. Likewise, Cdc11 subunits at the ends of adjacent yeast hetero-octamers interact via this same (so-called "NC") interface in filaments[9] [Fig. 1(B)]. We reasoned that dye-derivatized C43 might mimic Phe at this position and be well tolerated. Thus, once all other endogenous Cys were eliminated from Cdc11 and the other yeast septins, leaving C43 as the lone Cys in Cdc11 could provide a convenient site for

attachment of compatible FRET probes as reporters for *in vitro* rod–rod polymerization via this interface.

Genetic complementation by plasmid-encoded Cys-less septins

In otherwise wild-type cells, absence of any septin (except Shs1) is lethal, and partial loss-of-function mutations cause slower growth and hallmark changes in cell morphology, namely highly elongated buds and defects in localization of other septins. Therefore, we assessed the functionality of each Cys substitution by introducing the mutant septin on a low-copy-number centromeric (*CEN*) plasmid, and determining whether it rescued the defects manifest by a cell carrying a null allele (complete deletion) of the corresponding gene. For example, a *LEU2*-marked *CEN* plasmid (pJT3438) expressing Cdc3(C124V C253V C279V) was introduced into a haploid strain (YMVB33) carrying the *cdc3Δ0::kanMX* allele on chromosome XII and also expressing the wild-type *CDC3* gene from a *URA3*-marked *CEN* plasmid (pMVB100) to maintain cell viability. Leu^+ transformants were selected at 30ºC, passaged at the same temperature on solid medium containing 5-FOA to select for loss of the *URA3* plasmid, and scored for colonies able to grow, indicating that, when present as the sole source, Cys-less Cdc3 was able to support cell viability [Fig. 2(A)]. When viable clones were obtained, cell morphology was examined by light microscopy [Fig. 2(B)]. The same method was applied to Cys-less mutants of *CDC10*, *CDC11*, and *CDC12* [Fig. 2(B)]. For all four septins, each Cys-less variant, when plasmid expressed, was able to restore viability to the corresponding deletion mutant [Fig. 2(A) and data not shown] and supported normal morphology [Fig. 2(B)].

Absence of Shs1 does not prevent cell growth and causes only a mild, poorly penetrant change in cell morphology[23,49] that is exacerbated somewhat at a stressful temperature (37ºC).[50] Thus, functionality of Shs1 Cys substitutions was assessed by transforming a *CEN* plas-mid expressing each Shs1-GFP derivative in an *shs1*Δ strain (JTY2064), and examining Shs1-GFP localization at 37ºC. Wild-type Shs1-GFP localized to the bud neck in the expected patterns (single ring, hourglass-shaped collar, split rings) in the vast majority of cells [arrows, Fig. 2(C)]. Some cells displayed elongated buds and aberrant Shs1-GFP-containing structures away from the neck [arrowheads, Fig. 2(C)], indicating that, at elevated temperature, even wild-type Shs1-GFP is somewhat compromised for function due either to the GFP tag and/or to excess expression (i.e. greater abundance than when expressed from its chromosomal locus). In the vast majority of cells, Shs1(C29V)-GFP, Shs1(C148S)-GFP and Shs1(C29V C148S)-GFP localized in patterns indistinguishable from normal Shs1-GFP [Fig. 2(C)]. Thus, these Cys replacements did not prevent Shs1 interaction with other septins or assembly into higher-order structures at the bud neck. By contrast, a nonconservative substitution, Shs1(C29P)-GFP, although stably expressed, displayed diffuse fluorescence in every cell [Fig. 2(C)], indicating that this more drastic change crippled the folding and/or assembly competence of Shs1.

In summary, when expressed from its own promoter on a *CEN* plasmid, each of the five mitotic septins tolerated loss of all native Cys without noticeable effect on its function.

Genetic complementation by chromosomally expressed Cys-less septins

Mutations that modestly weaken binding affinity of a protein for its target or that reduce stability can sometimes be compensated for by overexpression from a plasmid. Hence, as an even more stringent test of functionality, each Cys-less septin variant was integrated into its normal chromosomal locus and expressed at its endogenous level. Furthermore, there are well-documented cases where the phenotypic defect of combining mutations in two different septins is much more drastic than each individual mutation alone (much more than additive),[43] especially mutations affecting guanine nucleotide binding. Bound nucleotide

induces conformational changes and contributes contacts that establish the "G" interface [Fig. 1(B)] between adjacent septin subunits.[9,17,43,46] When two, even nonadjacent septins lack nucleotide, hetero-octamer assembly and/or polymerization is incapacitated, especially at an elevated temperature well tolerated by normal cells. By the same logic, replacement of native Cys may subtly affect the conformation and/or stability of a septin in ways that are permissive when all other septins are wild-type, but may synergize deleteriously with Cys substitution mutations in another septin. Hence, we also assessed the effect of combining Cys-less variants of different septins in the same cell.

First, a C-terminally GFP-tagged version of Cdc10(C266S) was integrated at the *CDC10* locus on chromosome III and had no adverse effect (Table IV, and data not shown). Colony growth on solid agar medium at various temperatures was examined using serial dilutions of the mutant cells and was similar to the wild-type parental strain [BY4741 (*MAT***a**) or BY4742 (*MAT*α)] [Fig. 3(A), left panel]. Morphology of the mutant cells was also normal, compared to the wild-type control [Fig. 3(B), middle panel, and Table IV]. Moreover, Cdc10(C266S)-GFP localized properly to the bud neck [Fig. 3(B), middle panel]; but, as expected, the fluorescent signal was dimmer than plasmid-expressed Cdc10-GFP in control cells [Fig. 3(B), left panel].

Likewise, the Cys-less Cdc11 variant expressed from the *CDC11* locus on chromosome X had no adverse effect on growth (Table IV) or morphology [Fig. 3(B), right panel]. Similarly, double integrates expressing both Cdc10(C266S)-GFP and Cdc11(C43F C137A C138A) grew well at all temperatures [Fig. 3(A), middle panel], had a normal morphology, and localized the reporter septin at the bud neck properly [Fig. 3(B), third panel].

Cells expressing Cdc12(C40A C278S) from its locus on chromosome VIII, either alone [Fig. 3(C), second panel, and Table IV) or in combination with Cdc10(C266S)-GFP [Fig. 3(C), first panel] or Cdc11(C43F C137A C138A) [Fig. 3(A), right panel], were viable, but were clearly compromised in their growth rate and cellular morphology even at nonstressful temperatures. Moreover, the reporter septin was not properly localized at the presumptive bud neck in a significant fraction of these morphological aberrant cells [Fig. 3(C), first panel]. Another integrated Cdc12 variant with different Cys substitutions, Cdc12(C40V C278A), either alone or in combination with Cdc10(C266S)-GFP, behaved quite similarly to Cdc12(C40A C278S) (Table IV). Several corresponding single mutants— Cdc12(C40A), Cdc12(C40V), and Cdc12(C278S) —exhibited less severe phenotypes (Table IV), suggesting that each of the two Cys substitutions contributed incrementally to reducing Cdc12 functionality.

A similar, but more severe, picture emerged for Cdc3. Although plasmid-expressed Cdc3(C124V C253V C279V) fully complemented a *cdc3*Δ mutation [Fig. 2(A,B)], we were unable, despite repeated attempts, to isolate any viable integrant expressing the same Cys-less Cdc3 variant from its native promoter at the *CDC3* locus on chromosome XII. Likewise, we were unable to isolate viable integrants expressing Cdc3(C124V), Cdc3(C124S), Cdc3(C253V) or Cdc3(C279V) (Table IV). Thus, apparently, alteration of any Cys in Cdc3 markedly reduces its affinity for its partner septins and/or its stability, such that the pool of functional molecules of this subunit is only adequate *in vivo* at the level achieved by expression from a plasmid.

Properties of cells co-expressing Cys-less Cdc3, Cdc10, Cdc11, and Cdc12

Chromosomally expressed Cdc10(C266S)-GFP and chromosomally expressed Cdc11(C43F C137A C138A) appeared fully functional when produced alone or together (Fig. 3, and Table IV). When chromosomally expressed, either Cdc12(C40A C278S) or Cdc12(C40V C278A) also supported viability, although growth was clearly compromised (Fig. 3 and

Table IV). We used standard genetic methods to combine these three Cys-less septin alleles in the same cell. The resulting haploid strain expressing Cdc10(C266S)-GFP, Cdc11(C43F C137A C138A) and Cdc12(C40A C278S) as the sole sources of these septins, along with endogenous Cdc3, were capable of proliferating (albeit not robustly) at 26° and 30ºC (Table IV). The behavior of these triple mutants was not significantly different from cells expressing Cdc11(C43F C137A C138A) and Cdc12(C40A C278S) [Fig. 3(A)], but were significantly worse than cells expressing Cdc10(C266S)-GFP and Cdc12(C40A C278S) [Fig. 3(C) and Table IV]. Thus, Cys substitutions in Cdc11 and Cdc12 exhibit deleterious synergy, perhaps because these two subunits are nearest neighbors in the hetero-octamer [Fig. 1(B)].

Cells expressing Cys-less versions of Cdc10, Cdc11, and Cdc12 from their chromosomal loci were viable, as were *cdc3*Δ cells expressing plasmid-encoded Cys-less Cdc3. Hence, standard genetic methods were used to generate a haploid strain expressing all four Cys-less sep-tins. Presence of each Cys-less mutant as the sole source of the corresponding septin was confirmed by recovery and sequencing of the cognate coding regions from this strain (data not shown). Gratifyingly, cells expressing Cdc3(C124V C253V C279V) from the *CEN* plasmid and Cdc10(C266S)-GFP, Cdc11(C43F C137A C138A) and Cdc12(C40A C278S) from their chromosomal loci were alive [Fig. 3(C)] and grew well at low cultivation temperatures (26° and 30ºC) (Table IV). Therefore, no function of these septins that is essential for yeast cell viability requires a Cys residue in any of these four proteins. Moreover, expression of Cdc3(C124V C253V C279V) from the plasmid partially ameliorated the severe morphological defect displayed by cells expressing Cdc10(C266S)-GFP, Cdc11(C43F C137A C138A) and Cdc12(C40A C278S) [Fig. 3(C), right panel], perhaps explained by the fact that Cdc3 is a direct binding partner of Cdc12,[9] both via an NC interface [Fig. 1(B)] and via formation of a coiled-coil between their C-terminal extensions.[8,51] Hence, elevated expression of Cdc3(C124V C253V C279V) from the plasmid may favorably influence the conformation or stability of Cdc12(C40A C278S). Nonetheless, although viable and able to proliferate well, the cells expressing all four Cys-less septins displayed some morphological abnormality, and, in a significant fraction of the population, Cdc10(C266C)-GFP was not properly localized at the presumptive bud neck [Fig. 3(C), right panel].

Steady-state expression level of Cys-less septins

Cys substitutions could deleteriously impact folding, stability or solubility of a septin, rather than interfere with hetero-octamer formation and function. Alternatively, Cys replacements might not affect folding, stability, or solubility, but could instead alter conformation in ways that affect subunit affinity for hetero-octamer assembly and function. In principle, the latter type of defect should be overcome by mass action, *i.e*. by providing more of the mutant protein, perhaps explaining why each Cys-less septin rescued the corresponding null mutation when expressed from a plasmid. *CEN* plasmids are typically present at ~3 copies per cell (because their mitotic transmission fidelity is inferior to that of full-length chromosomes); if the marker (or the gene) carried is not fully functional, cultivation will select for cells with an even more elevated plasmid copy number, yielding lineages with plasmid copy numbers of 5–10 per cell.[52] In a *cdc3*Δ or *cdc12*Δ strain, where expression of *CDC3* or *CDC12*, respectively, is essential for viability, the plasmids encoding Cdc3(C124V C253V C279V) or Cdc12(C40A C278S) will be under strong selective pressure to amplify their copy number, potentially masking the true degree of dysfunctionality of these mutant proteins. Conversely, it is much harder to amplify the entire chromosome (or chromosomal region) when a hypomorphic Cys-less septin mutant is integrated at its chromosomal locus, and the dysfunctional nature of the mutant protein is revealed because it cannot be expressed at a level sufficient to efficiently assemble septin complexes and higher-order structures.

To explore these issues, we used immunoblotting to compare the steady-state level of each normal septin and its Cys-less variant expressed from its chromosomal locus. Wild-type Cdc11 and Cdc11(C43F C137A C138A) in cells in which the nature of the other septins was varied (except for endogenous Cdc3, which served as the loading and normalization control) were examined first. Regardless of whether another septin(s) was also altered, the steady-state level of wild-type Cdc11 was reproducibly higher than Cdc11(C43F C137A C138A) (Fig. 4, top panel, lane 1 versus lane 4, lane 3 versus lane 6, and lane 5 versus lane 7). Likewise, regardless of strain background, the steady-state level of wild-type (untagged) Cdc10 was always higher than that of Cdc10(C266S)-GFP (Fig. 4, second panel). In this case, presence of the GFP tag (and/or downstream DNA sequences associated with this fusion), rather than the C266S substitution, could be responsible for the difference. However, we always chose for Cys replacements the most frequently used triplet (based on yeast codon usage bias[53]) to encode the residue substituted. It is unlikely, therefore, that the observed reduction in Cys-less Cdc10 and Cdc11 compared to their wild-type counterparts was due to less efficient translation. We did not assess the level of each corresponding mRNA, so we cannot rule out that the base pairs changes destabilized the transcripts of the mutant proteins somewhat. Nevertheless, because cells chromosomally expressing Cdc10(266S)-GFP or Cdc11(C43F C137A C138A) [Fig. 3(B)], or both [Fig. 3(A,C)], exhibited no defects in proliferation, morphology, or localization of the reporter septin (summarized in Table IV), the observed decreases in the cellular concentrations of Cdc10 and Cdc11 were clearly in a well tolerated range.

The influence of other Cys-less septins in the same cell was not necessarily deleterious for any other Cys-less sep-tin. For example, focusing on Cdc11(C43F C137A C138A) itself, its level was elevated up to about two-fold in cells expressing Cdc12(C40A C278S) as compared to cells expressing wild-type Cdc12 (Fig. 4, top panel, lanes 2 and 4 versus lanes 6 and 7). Likewise, presence of either Cdc11(C43F C137A C138A) or Cdc12(C40A C278S), or both, did not have any marked effect on the level of Cdc10(C266S)-GFP (Fig. 4, second panel, lanes 5, 6, and 7).

As observed for Cdc10 and Cdc11, regardless of strain background, the level of wild-type Cdc12 was always higher than that of Cdc12(C40A C278S) (Fig. 4, third panel, lane l versus lane 6, lane 2 versus lane 5, lane 3 versus lane 7). The lowest level of Cdc12(C40A C278S) was observed in cells also expressing Cys-less Cdc10 and Cys-less Cdc11 (Fig. 4, third panel, lane 7), likely explaining the pronounced growth phenotype of those cells. Under-expression of Cdc12 should be poorly tolerated, for several reasons. We showed previously[23] that yeast cells can tolerate loss of Cdc11 (provided that Shs1 is also absent) because the resulting Cdc12-capped hetero-hexamers can polymerize into filaments via non-native Cdc12-Cdc12 contacts mediated by their G interface [Fig. 1(B)]. If the level of Cdc12 is low relative to the other septins, neither normal hetero-octamers nor polymerization-competent hetero-hexamers will form efficiently and, consequently, both filament formation and cytokinesis will fail.

Similarly, regardless of strain background, and normalized to the level of any other wild-type septin present in the same cell (Cdc11, in the case of the representative example shown), the level of plasmid-expressed wild-type Cdc3 was always higher than that of plasmid-expressed Cdc3(C124V C253V C279V) (Fig. 4, bottom panel). As we also showed previously,[23] yeast cells can tolerate loss of Cdc10 because Cdc11-capped hetero-hexamers can form via non-native Cdc3-Cdc3 contacts mediated by their G interface, which are able to polymerize into filaments via the normal Cdc11-Cdc11 NC interface [Fig. 1(B)]. If the level of Cdc3 is low relative to the other septins, neither hetero-octamers nor such polymerization-competent hetero-hexamers can form efficiently and, consequently, filament

formation and cytokinesis both fail. Hence, as for Cdc12, under-expression of Cdc3 should be poorly tolerated.

The above considerations and the results of the analyses shown in Figure 4 likely explain why the Cys-less mutant allele Cdc12 was unable to fully rescue septin function when expressed from its corresponding chromosomal locus and why the Cys-less mutant allele of Cdc3 when expressed from its chromosomal locus was unable to support viability, whereas both Cdc3(C124V C253V C279V) and Cdc12(C40A C278S) expressed from plasmids supported normal growth. It is especially striking that cells expressing Cdc3(C124V C253V C279V) from a plasmid and chromosomally expressing Cys-less Cdc10, Cdc11, and Cdc12 grow remarkably well, further emphasizing the importance of producing enough of each sep-tin to achieve the correct stoichiometry of subunits *in vivo*.

Hetero-octamer assembly and filament formation by recombinant Cys-less septins

The above findings indicated that differences in expression level and not any other intrinsic defects are largely responsible for the observed deficiencies in functional complementation *in vivo* by Cys-less Cdc3 and Cdc12. As the most stringent test of this conclusion, we produced all the Cys-less septins as recombinant proteins by co-expression in *E. coli* and examined their properties and functionality *in vitro*. We have described in detail elsewhere[8–10] the vectors and induction system for heterologous expression and purification of septins and sep-tin mutants in bacterial cells, where their presence has no phenotypic consequences on which selection could act. Co-expression of wild-type Cdc3, Cdc10, Cdc11, and $(His)_6$-Cdc12 in *E. coli* generates hetero-octameric complexes with a 2:2:2:2 stoichiometry[9,10] that are stable in high salt (≥0.2*M* KCl) [Fig. 5(A), left panel, inset] and able, when the salt concentration is reduced (≤50 m*M* KCl), to polymerize into long paired filaments readily visualized by EM [Fig. 5(A), left panel]. As for wild-type septins, co-expression of Cdc3(C124V C253V C279V), Cdc10(C266S), Cdc11(C43F C137A C138A) and $(His)_6$-Cdc12(C40A C278S) yielded hetero-octameric complexes in a 2:2:2:2 stoichiometry that were stable in high salt [Fig. 5(A), middle panel, inset]. Under EM, the particles present were ~32 x ~4 nm rods indistinguishable in dimensions from those composed of wild-type septins (data not shown). As judged by size exclusion chromatography and compared to the relative abundance of hetero-octamers in preparations of wild-type septin complexes, we did not observe any increase in sub-octameric species in our preparations of complexes composed of Cdc3(C124V C253V C279V), Cdc10(C266S), Cdc11(C43F C137A C138A) and $(His)_6$-Cdc12(C40A C278S). In fact, no increases in sub-octameric species were observed with any other Cys-less mutant complex we analyzed (see below). Thus, hetero-octamers composed of the Cys-less septins did not appear more fragile.

On the other hand, overall yield of the Cys-less septin complex was always lower than for wild-type, yielding preparations with a lower final protein concentration, which, on first principles and in our experience, reduces the efficiency of filament formation.[9] Indeed, on standard carbon-coated EM grids, filament formation in low salt from such dilute Cys-less septin complexes was less robust [Fig. 5(A), middle panel]. However, as we demonstrated previously,[54] an EM grid coated with a lipid monolayer containing phosphatidylinositol-4,5-bisphosphate (PIP_2) reduces dramatically the critical protein concentration required for filament formation (relative to that in solution). Hence, instead of using biochemical methods to concentrate the solution containing the Cys-less septin complex, we transferred them into low-salt buffer on EM grids coated with a PIP_2-containing lipid monolayer. Reassuringly, the Cys-less septin complexes displayed robust formation of long paired filaments equivalent to those observed for wild-type complexes under the same conditions [Fig. 5(A), right panel]. Thus, when co-expressed (even in a heterologous host cell), the four Cys-less septins are stably produced in soluble form, competent to assemble into

stoichiometric hetero-octamers, and fully capable of polymerizing into filaments. Thus, each Cys-less septin is able to fold and interact with its partner septins efficiently, confirming that the genetic complementation defects observed *in vivo* for Cdc3 and Cdc2 were likely just a consequence of sub-optimal expression.

Having successfully removed all endogenous Cys from the four major mitotic septins, we next tested whether it was possible to introduce single Cys substitutions at new locations in specific septins, in particular whether Cys installed at sites predicted by structural modeling to lie near particular septin-septin interfaces [Fig. 1(B)] would be tolerated. In crystal structures of corresponding human septins,[45,46,48,55] there is a solvent-exposed residue located near the end of helix α6 close to residues that constitute the NC interface and which, in Sept6, is natively a Cys (C301) [Fig. 1(C)], indicating that Cys is acceptable at this position. Moreover, in Sept6, Cys301 does not make direct contact with the adjacent monomer and, hence, its modification may not disrupt the NC interface. Therefore, we mutated to Cys the corresponding residue in each Cys-less septin— Ser407Cys in Cdc3(C124V C253V C279V), Arg298Cys in Cdc10(C266S), Glu294Cys in Cdc11(C43F C137A C138A) and Leu310Cys in Cdc12(C40A C278S) [Fig. 1(C)]. Each such Cys "add back" mutant was co-expressed in *E. coli* with the three other Cys-less septins and all efficiently formed recombinant hetero-octamers that were stable in high salt [Fig. 5(B), insets] and capable of robust filament polymerization in low salt [Fig. 5(B)] even on standard carbon-coated grids (because the protein concentration of these purified preparations was quite high). Thus, Cys can indeed be introduced at new positions into otherwise Cys-less septin variants without disturbing either their ability to assemble into hetero-octamers or their ability to polymerize into filaments. The ability to engineer thiol groups at unique positions in these proteins provides access to new tools for sulfhydryl-based attachment of various types of chemical substituents that should be useful for on-going analysis of the structure and function of these cytoskeletal polymers.

ADDENDUM

During the site-directed mutagenesis required for construction of the otherwise Cys-less mutant Cdc11(E294C), a primer sequence was inadvertently incorporated into the PCR product after nucleotide 870 (the last base of the codon specifying Arg290). Presence of this insert allowed the open-reading frame to continue correctly (including the E294C substitution) up to and including Ala302, but then added 25 residues of non-native sequence (-RYYMK DIEPVHYQVNLSRQNPYVQI-COOH) before encountering a stop codon (TGA), in effect truncating the protein (removing residues 303-to-415). However, we have demonstrated previously that a Cdc11 mutant deleted for its entire C-terminal extension, Cdc11(Δ306–415), forms hetero-octamers and polymerizes into paired filaments with essentially wild-type efficiency both in solution and on the PtdIns4,5P_2-containing monolayers [Fig. 3a in Bertin et al. (2010) *J. Mol. Biol.*]. Likewise, as we demonstrated here in Fig. 5, Cdc11(C43F C137A C138A E294C, Δ303–415) assembled efficiently into hetero-octamers that retained the capacity for filament formation.

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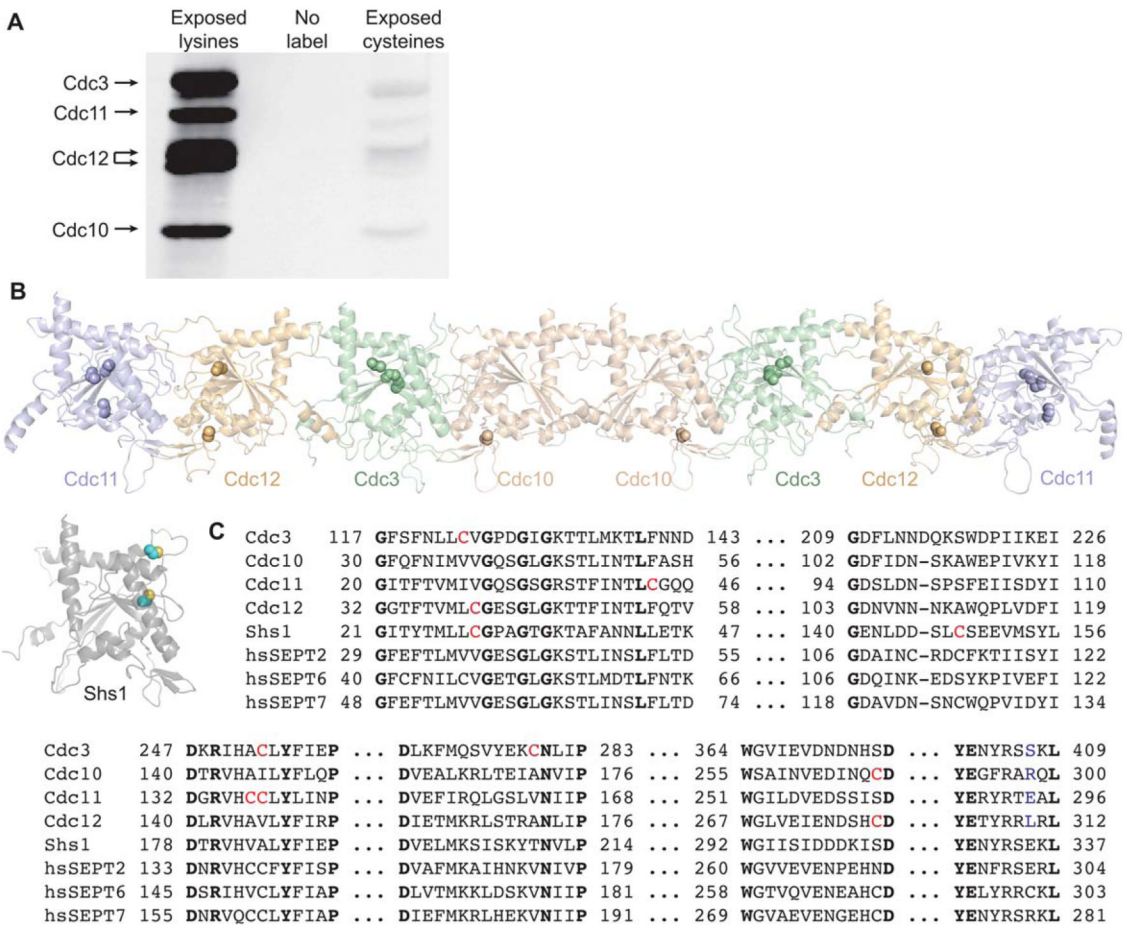

Figure 1.

Location and conservation of cysteine residues in yeast septins. (**A**) Purified recombinant Cdc11–$(His)_6$-Cdc12–Cdc3–Cdc10–Cdc10–Cdc3–$(His)_6$-Cdc12–Cdc11 hetero-octamers (10 μg) were incubated in buffer alone (No label) or exposed to a biotinylated compound containing a moiety reactive either with primary amines (Exposed lysines) or with thiols (Exposed cysteines), as described in Materials and Methods section, and the resulting products were resolved by SDS-PAGE in a 10% gel, transferred to a nitrocellulose membrane, detected by incubation with infrared dye-labeled streptavidin, and analyzed using an infrared imager. (**B**) Homology model[54] of Cdc11–Cdc12–Cdc3–Cdc10–Cdc10–Cdc3–Cdc12–Cdc11 hetero-octamer (top), or with alternative terminal subunit Shs1[10] (bottom), and with endogenous cysteine residues depicted as space-filling spheres. The central Cdc10-Cdc10 pair are joined by a so-called NC interface; each Cdc10 is joined to its neighboring Cdc3 by a so-called G interface (guanine nucleotides not shown); each Cdc3 is joined to its adjacent Cdc12 by an NC interface; each Cdc12 is joined to its juxtaposed Cdc11 by a G interface; and, thus, the Cdc11-Cdc11 interaction responsible for the polymerization of hetero-octameric rods into filaments is an NC interface. (**C**) Sequence alignment of relevant portions of the *S. cerevisiae* mitotic septins and the human septins used for the structural homology models shown in (B). *Bold*, residues conserved in all eight septins. Red, endogenous Cys in yeast septins. Blue, residues substituted to Cys in otherwise Cys-less recombinant yeast hetero-octamers.

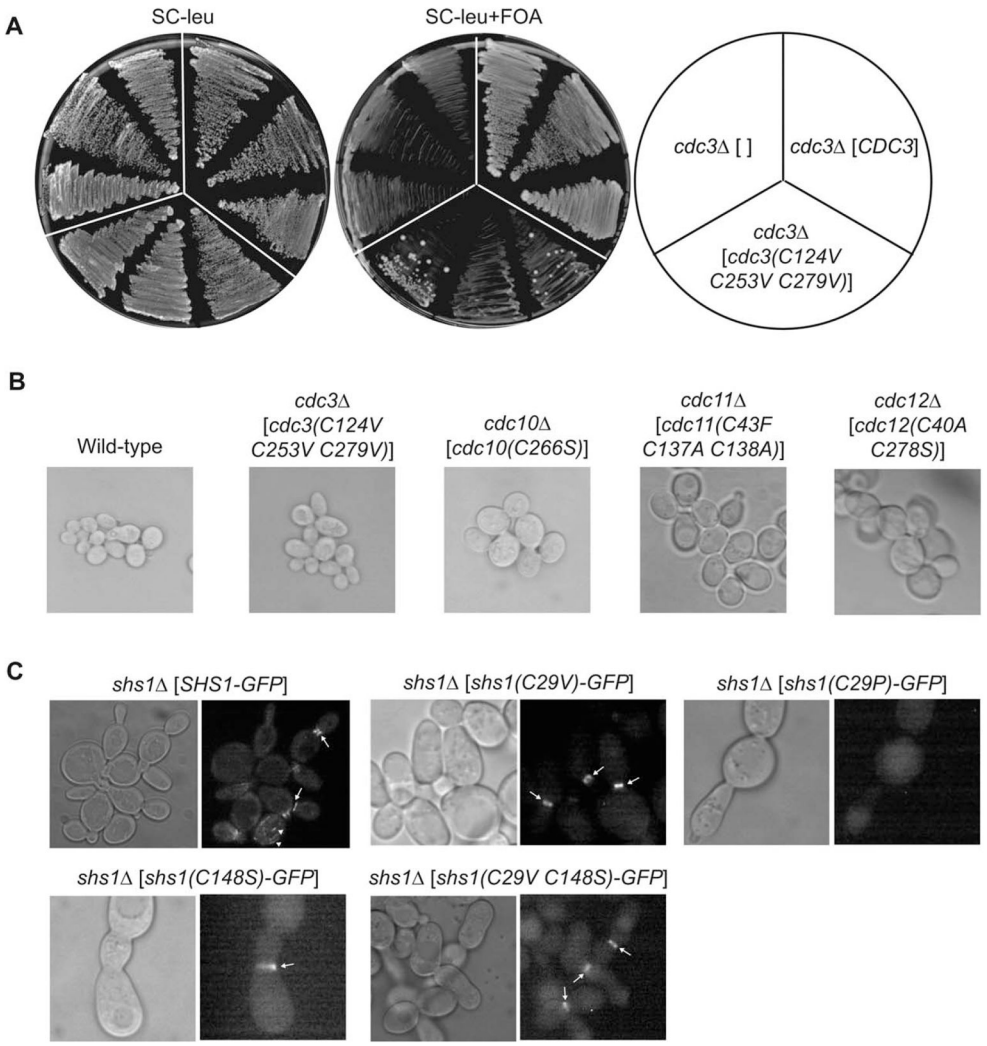

Figure 2.

Complementation by plasmid-expressed Cys-less septins. (**A**) A yeast strain (YMVB33) harboring a chromosomal deletion of *CDC3* and carrying a *URA3*-marked plasmid encoding wild-type *CDC3* was transformed with an empty *LEU2*-marked vector (empty brackets) or the same vector carrying either normal *CDC3* or expressing a Cys-less Cdc3 derivative [*cdc3(C124V C253V C279V)*] and Leu^+ transformants were selected. Three independently obtained Leu^+ transformants of the genotypes indicated to the right were then streaked onto synthetic complete medium lacking leucine (SC-leu) (*left*) or the same medium containing 5-fluoro-orotic acid (*middle*) to select for cells that have lost the *URA3*-marked plasmid, and the plates were photographed after 3 days of incubation at 30ºC. (**B**) The same plasmid shuffle strategy described in (A) was used to isolate BY4741-derived strains containing the indicated septin gene deletion mutation and expressing the corresponding Cys-less septin mutant from a *LEU2*-marked *CEN* plasmid: *cdc3*Δ [*cdc3(C124V C253V C279V)*] (FOA-resistant isolate of YMVB33 transformed with pJT3438); *cdc10*Δ [*cdc10(C266S)*] (FOA-resistant isolate of JTY4203 transformed with pJT3448); *cdc11*Δ [*cdc11(C43F C137A C138A)*] (FOA-resistant isolate of SBY110 transformed with pJT3283); and *cdc12*Δ [*cdc12(C40A C278S)*] (FOA-resistant isolate of YMVB12 transformed with pJTY3566). Single colonies of these strains, and wild-type control cells (BY4741), were inoculated into SC-leu, grown to mid-exponential phase at 30ºC, and samples were photographed using

transmitted light microscopy. (**C**) A derivative of strain BJ2168 harboring an *shs1*Δ mutation was transformed with *TRP1*-marked *CEN* plasmids expressing the Shs1 variants containing the indicated Cys replacement mutations, namely *SHS1-GFP* (pRS314-SHS1-GFP), *shs1(C29V)-GFP* (pJT3126), *shs1(C148S)-GFP* (pJT3154), *shs1(C29V C148S)-GFP* (pJT3155), and *shs1(C29P)-GFP* (pJT3125), and the resulting transformants were grown at 37ºC in SC-trp to mid-exponential phase, and then samples of each culture were examined by both transmitted light (left panels) and epifluorescence (right panels) microscopy.

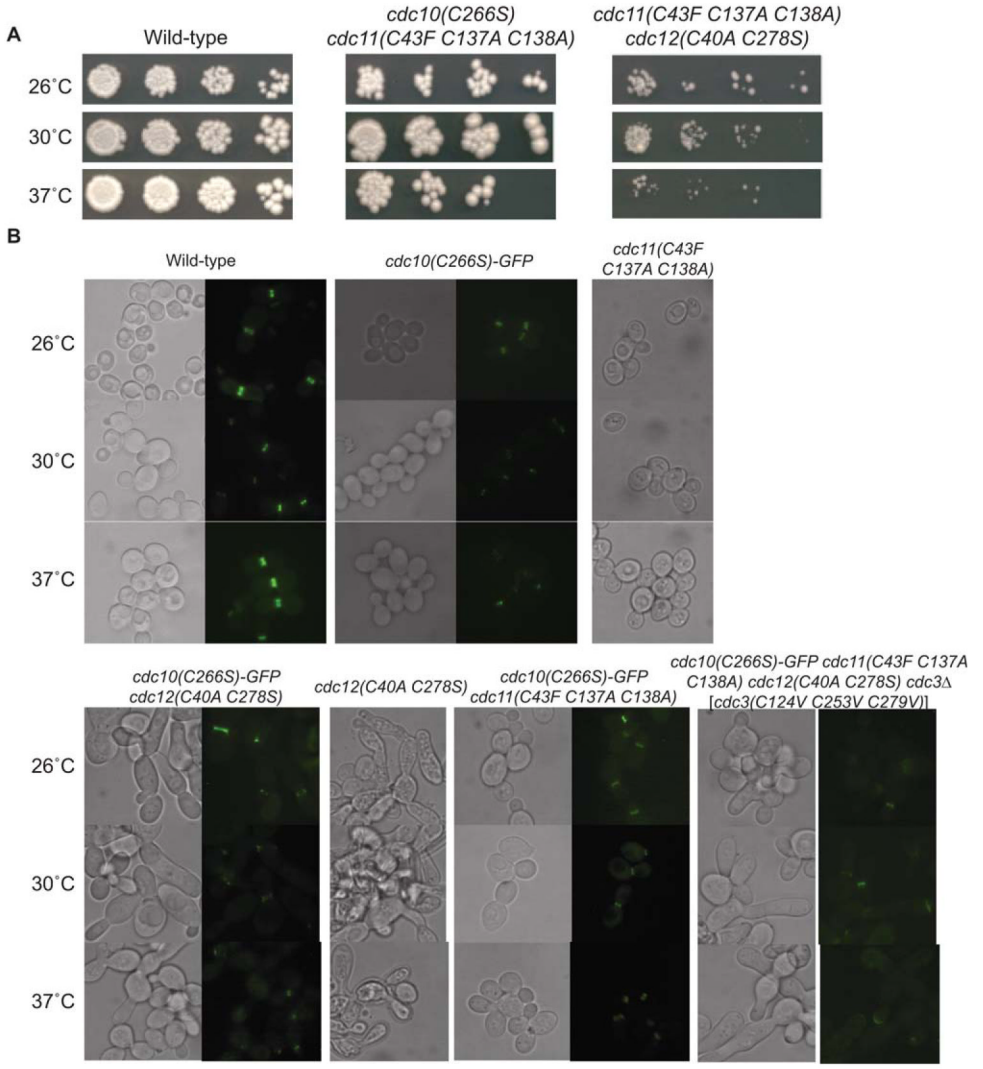

Figure 3.

Complementation by chromosomally expressed Cys-less septins. (**A**) To assess growth rate, otherwise isogenic control cells (wild-type, BY4742) and the indicated double integrant strains, *cdc10(C266S)-GFP cdc11(C43F C137A C138A)* (JTY5019) and *cdc11(C43F C137A C138A) cdc12(C40A C278S)* (JTY5018), were grown to mid-exponential phase in rich liquid medium (YPD), adjusted with fresh medium to the same approximate turbidity, and equal volumes (5 μL) of the initial sample and three serial 10-fold dilutions were spotted onto YPD plates and photographed after 3 days of incubation at the indicated temperature. (**B**) To assess cellular morphology and localization of the reporter septin [Cdc10-GFP or Cdc10(C266S)-GFP], if present, strains of the indicated genotype, namely, wild-type (BY4741 [pLA10K]); *cdc10(C266S)-GFP* (JTY4984); *cdc11(C43F C137A C138A)* (JTY5001); *cdc10(C266S)-GFP cdc12(C40A C278S)* (JTY4992); *cdc12(C40A C278S)* (JTY4998); *cdc10(C266S)-GFP cdc11(C43F C137A C138A)* (JTY5019); and, *cdc10(C266S)-GFP cdc11(C43F C137A C138A) cdc12(C40A C278S) cdc3Δ0* [*cdc3(C124V C253V C279V)*] (JTY5043), were cultivated in YPD to mid-exponential phase at the indicated temperature and then examined by transmitted light microscopy (left) and, where indicated, by epifluorescence microscopy (right). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

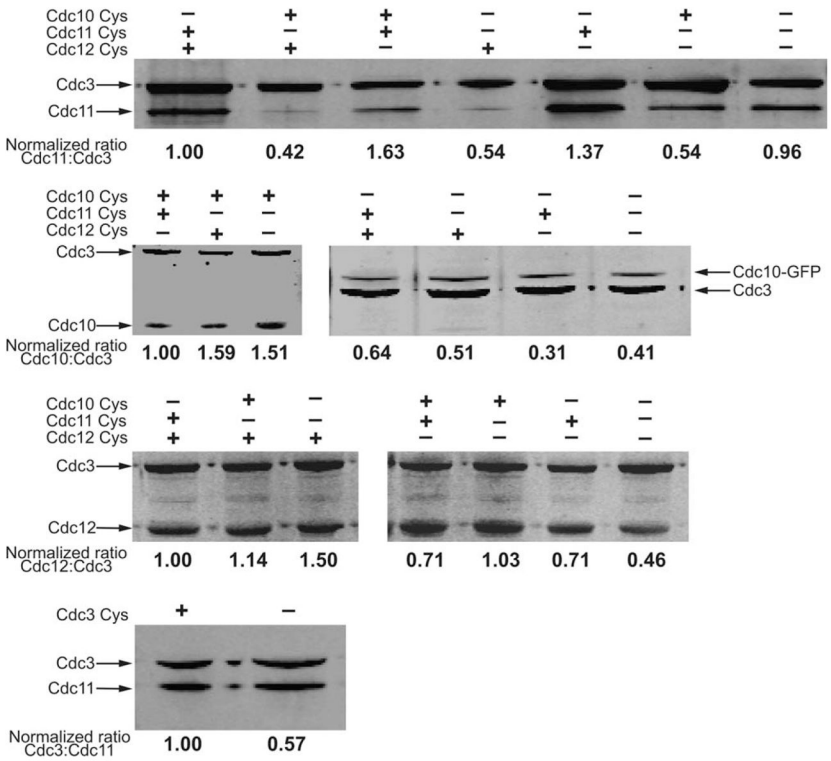

Figure 4.

Analysis of the relative abundance of Cys-less septins. Top three panels, otherwise isogenic strains expressing from the corresponding chromosomal locus either the wild-type locus for the indicated septin (+) or the Cys-less allele of the indicated septin (−) were grown to mid-exponential phase in YPD, harvested, lysed, and equivalent samples (~30 μg total protein) were resolved by SDS-PAGE and analyzed by immunoblotting with the appropriate pair of primary anti-septin antibodies and secondary antibodies labeled with an infrared dye, as described in Materials and Methods. In all strains examined, wild-type Cdc3 expressed from its endogenous chromosomal locus was used as a loading control to normalize the level of the other septin analyzed; the value below each lane represents the ratio of the background-corrected signal for the indicated septin to that for Cdc3. Bottom panel, as above, except that wild-type and Cys-less Cdc3 were plasmid-expressed and normalized to the level of chromosomally expressed Cdc11. Strains examined included: JTY4984, JTY5001, JTY4998, JTY4992, JTY5019, JTY5018, JTY5003, and FOA-resistant isolates of YMVB33 transformed with pMVB100 and pJT3438, respectively.

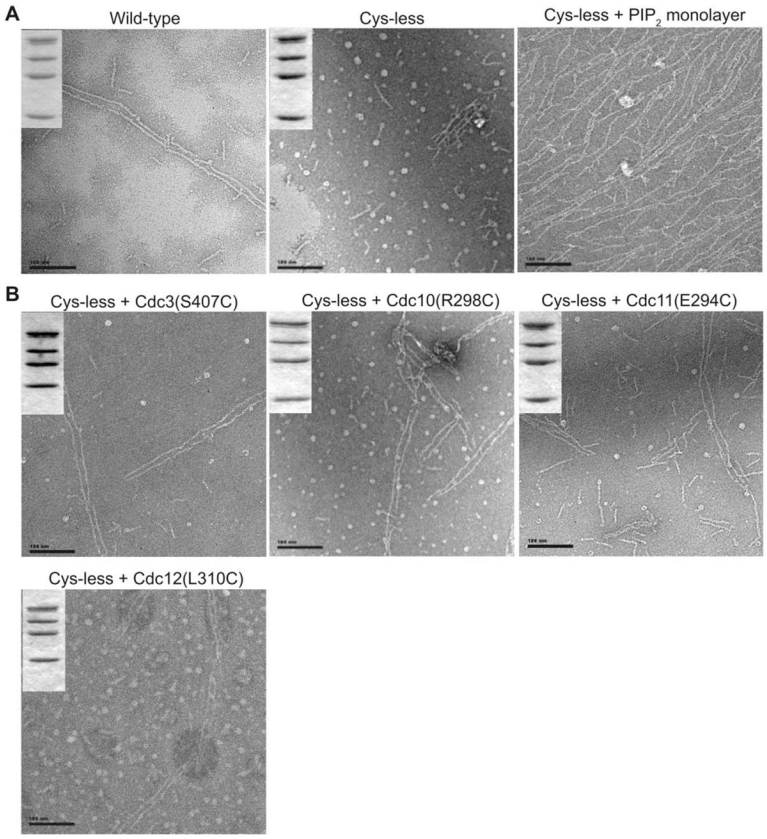

Figure 5.

Assembly, purification, and filament formation properties of recombinant Cys-less septin hetero-octamers. (**A**) Either wild-type Cdc3, Cdc10, and Cdc11 were co-expressed with $(His)_6$-Cdc12 in *E. coli*, or the Cys-less versions of these septins were co-expressed in the same way, and the cells lysed in high-salt buffer, and the resulting complexes were purified using a three-step procedure (immobilized metal ion chromatography, size exclusion chromatography, and ion exchange chromatography). Samples (~20 μg total protein) of the purified complexes obtained were resolved by SDS-PAGE and analyzed by staining with Coomassie blue (insets) and other aliquots were diluted into a low-salt buffer and after 1 h deposited on standard carbon-clad grids (left and middle) or on the same type of grid coated with an artificial lipid monolayer composed of dioleoyl-phosphatidylcholine containing 20% phosphatidylinositol-4,5-bisphosphate (PIP_2) (right) and visualized by EM. (**B**) As in (A); but, in each case, the otherwise Cys-less version of the indicated septin containing a novel Cys substitution at the indicated position was co-expressed with the Cys-less versions of the other three septins. The circular “blobs” present to a greater or lesser extent in some of the preparations are a large (420,000), endogenous, homohexameric *E. coli* Ni^{2+}-binding enzyme (ArnA) that co-purifies with septin complexes to a variable extent from preparation to preparation. Scale bars, 100 nm.

Table I

Yeast Strains Used in This Study

Strain	Genotype	Reference/source
BY4741	***MAT*a *his3*Δ1 *leu2*Δ0 *met15*Δ0 *ura3*Δ0**	56
BY4742	***MAT*α *his3*Δ1 *leu2*Δ0 *lys2*Δ0 *ura3*Δ0**	56
JTY3647	***MAT*a/*MAT*α *his3*Δ*1/his3*Δ*1 leu2*Δ*0/leu2*Δ*0 met15*Δ*0/MET15 ura3*Δ*0/ura3*Δ*0 lys2*Δ*0/LYS2 cdc3*Δ*0::KanMX/CDC3***	American Type Culture Collection
YMVB33	***MAT*a *his3*Δ*1 leu2*Δ*0 MET15*$^{+}$ *cdc3*Δ*0::KanMX* [*CDC3 URA3*]**	23
JTY5022[a]	***met15*Δ*0 cdc3*Δ*0::KanMX* [*cdc3(C124V C253V C279V) MET15*]**	This work
4003482™	BY4741 ***cdc10*Δ*0::kanMX4***	American Type Culture Collection
JTY4203	***MAT*a *ura3*Δ*0 met15*Δ*0 his3*Δ*1 leu2*Δ*0 cdc10*Δ*::KanMX* [*CDC10 URA3*]**	23
YMVB61	***MAT*a *ura3*Δ*0 leu2*Δ*0 cdc12*Δ*::KanMX* [*CDC12 URA3*]**	23
BYB67	***MAT*a/*MAT*α *ade2-1/ADE2 his3–11,5/his3–11,15 leu2–3,112/leu2–3,112 ura3-1/ura3-1 lys2*Δ*::hisG/LYS2 trp1-1/trp1-1***	56
SBY110[b]	***MAT*a *can1–100 his3–11,15 leu2–3,112 trp1-1 ura3-1 cdc11*Δ*::HIS3* [*CDC11 URA3*]**	Shirin Bahmanyar (this laboratory)
JTY4984[c]	BY4741 ***cdc10(C266S)-GFP::KanMX***	This work
JTY5001[d]	BY4742 ***cdc11(C43F C137A C138A)::URA3***	This work
JTY5002[d]	BY4742 ***cdc11(C137A C138A)::URA3***	This work
JTY4992[e]	BY4741 ***cdc10(C266S)- GFP::KanMX cdc12(C40A C278S)::LEU2***	This work
JTY4998[e]	BY4741 ***cdc12(C40A C278S)::LEU2***	This work
JTY4999[e]	BY4741 ***cdc12(C40A)::LEU2***	This work
JTY5000[e]	BY4741 ***cdc12(C278S)::LEU2***	This work
JTY5018[f]	BY4741 ***cdc11(C43F C137A C138A)::URA3 cdc12(C40A C278S)::LEU2***	This work
JTY5019[f]	BY4741 ***cdc11(C43F C137A C138A)::URA3 cdc10(C266S)-GFP::KanMX***	This work
JTY5017[f]	BY4742 ***cdc10(C266S)-GFP::KanMX cdc11(C43F C137A C138A)::URA3 cdc12(C40A C278S)::LEU2***	This work
JTY5003[f]	BY4741 ***cdc10(C266S)-GFP::KanMX cdc11(C43F C137A C138A)::URA3 cdc12(C40A C278S)::LEU2***	This work
JTY5028[e]	BY4742 ***cdc12(C40V)::LEU2***	This work
JTY5043[g]	BY4741 ***cdc10(C266S)-GFP::KanMX cdc11(C43F C137A C138A)::URA3 cdc12(C40A C278S)::LEU2 cdc3*Δ*0::KanMX* [*cdc3(C124V C253V C279V) MET15*]**	This work
BJ2168	***prb1–1122 pep4-3 pre1–451 gal2***	57
JTY2064[h]	BJ2168 ***shs1*Δ*0::kanMX4***	Björn Gullbrand (this laboratory)

[a] Derived from a spore clone of strain JTY3647 that was, prior to sporulation, transformed with plasmid pJT2059. The spore clone was transformed with plasmid pJT3523, and loss of pJT2059 was selected with FOA.

[b] Spore clone of strain BYB67 that was, prior to sporulation, transformed with plasmid pSB3.

[c] A PCR product containing a *cdc10(C266S)-GFP::KanMX* cassette was amplified using template plasmid pJT3797 and primers cdc10seq2 and cdc10RS- and transformed into strain BY4741.

[d] A PCR product containing a *cdc11(C43F C137A C138A)::URA3* cassette was amplified using template plasmid pJT3518 and primers cdc11FWD-new and cdc11RS- and transformed into strain BY4741 or BY4742.

[e] A PCR product containing a cassette with *LEU2* and a Cys-mutant allele of *CDC12* was amplified in two steps. First, a PCR product containing *CDC12* sequences including and 3′ of the Cys mutations was amplified using template plasmids pJT3279, pJT3282, pJT3563, or pJT3566 and primers 12GEXFW and Duet12XhoIre. In parallel, a PCR product containing *LEU2* and a short stretch of homology with the 3′ end of *CDC12* was amplified using template plasmid pMVB45 and primers cdc12endRS+new and cdc12term_RS-. Second, a PCR product effectively joining these two fragments was amplified using primers 12GEXFW and cdc12term_RS-.

[f] Spore clone from a cross between JTY5001 and JTY4992.

[g] Spore clone from a cross between JTY5003 and JTY3647.

[h] A PCR product containing the *KanMX* gene flanked by homology to the sequences surrounding the *SHS1* ORF was amplified using template plasmid pFA6a-kanMX4 and primers shs1dfw and shs1dre and transformed into strain BJ2168.

Table II

Plasmids Used in This Study

Plasmid	Relevant properties	Source/reference
pFA6a-kanMX	***KanMX***	58
pRS400	***KanMX***	59
pRS421	2μ, ***MET15***	59
pRS426	***URA3***	59
pMVB100	***CEN***, ***URA3***, ***CDC3***	8
pMVB45	***CEN***, ***LEU2***, ***CDC12***	8
p315-CDC3-HA	***CEN***, ***LEU2***, ***CDC3-HA***	60
pJT3564[a]	***CEN***, ***MET15***, ***CDC3-HA***	This study
pMVB166[b]	***CEN***, ***HIS3***, ***CDC10***	Mathias Versele (this laboratory)
pLA10K[c]	***CEN***, ***KanMX***, ***CDC10-GFP***	This study
pJT3448[d]	pMVB166 ***cdc10(C266S)***	This study
pJT3797[d]	pLA10 ***cdc10(C266S)-GFP***	This study
pSB3	***CEN***, ***TRP1***, ***CDC11***	Shirin Bahmanyar (this laboratory)
pJT3277[d]	pSB3 ***cdc11(C43F)***	This study
pJT3283[d]	pJT3277 ***cdc11(C43F C137A C138A)***	This study
pJT3518[e]	pJT3283 ***URA3***	This study
pRS314-SHS1-GFP	***CEN, TRP1, SHS1-GFP***	50
pJT3126[d]	pRS314-SHS1-GFP ***shs1(C29V)-GFP***	This study
pJT3125[d]	pRS314-SHS1-GFP ***shs1(C29P)-GFP***	This study
pJT3154[d]	pRS314-SHS1-GFP ***shs1(C148S)-GFP***	This study
pJT3155[d]	pRS314-SHS1-GFP ***shs1(C29V C148S)-GFP***	This study
pJT3279[d]	pMVB45 ***cdc12(C40A)***	This study
pJT3282[d]	pJT3279 ***cdc12(C40A C278S)***	This study
pJT3563[d]	pMVB45 ***cdc12(C40V)***	This study
pJT3566[d]	pJT3563 ***cdc12(C40V C278A)***	This study
pJT3436[d]	p315-CDC3-HA ***cdc3(C124V)-HA***	This study
pJT3437[d]	pJT3436 ***cdc3(C124V C253V)-HA***	This study
pJT3438[d]	pJT3437 ***cdc3(C124V C253V C279V)-HA***	This study
pJT3523[f]	***CEN***, ***MET15***, ***cdc3(C124V C253V C279V)-HA***	This study
pJT3567[d]	pJT3564 ***cdc3(C124A)-HA***	This study
pJT3663[d]	pJT3564 ***cdc3(C124S)-HA***	This study
pMVB128	ColE1 ori, Amp^R, ***lacI, P_{T7}–His_6-CDC12, P_{T7}–CDC10***	Mathias Versele (this laboratory)
pJT3518[d]	pMVB128 ***cdc10(C266S) cdc12(C40A C278S)***	This study
pMVB133	P15a ori, $Chlor^R$, ***lacI, P_{T7}–CDC3, P_{T7}–CDC11***	Mathias Versele (this laboratory)
pJT3636[d]	pMVB133 ***cdc3(C124V C253V C279V) cdc11(C43F C137A C138A)***	This study

Plasmid	Relevant properties	Source/reference
pJT3620[d]	pJT3620 ***cdc3(C124V C253V C279V S407C)***	This study
pJT3612[d]	pJT3518 ***cdc10(C266S R298C)***	This study
pJT3611[d]	pJT3518 ***cdc12(C40A C278S L310C)***	This study
pJT3621[d]	pMVB133 ***cdc11(C43F C137A C138A E394C)***	This study

[a] A cassette encoding the *MET15* marker gene and flanking regions was amplified by PCR with template plasmid pRS421 and primers pRS_5 and pRS_3 and used to co-transform a yeast strain (BY4741) with p315-CDC3-HA, the *LEU2* marker gene of which harbors identical flanking regions, allowing for recombination-mediated marker exchange *in vivo*.

[b] Includes *CDC10* promoter and ~330 bp downstream of *CDC10* ORF.

[c] A cassette encoding the *KanMX* marker gene (conferring G418 resistance) and flanking regions was amplified by PCR with template plasmid pRS400 and primers pRS_5 and pRS_3 and used to transform a yeast strain (BY4741) carrying pLA10, the *URA3* marker gene of which harbors identical flanking regions, allowing for recombination-mediated marker exchange *in vivo*.

[d] The parent plasmid was used as a template with appropriate primer pairs (Table III) to introduce by site-directed mutagenesis the indicated Cys mutations.

[e] A cassette encoding the *URA3* marker gene and flanking regions was amplified by PCR with template plasmid pRS426 and primers pRS_5 and pRS_3 and used to transform a yeast strain (BY4741) carrying pJT3283, the *TRP1* marker gene of which harbors identical flanking regions, allowing for recombination-mediated marker exchange *in vivo*.

[f] A cassette encoding the *MET15* marker gene and flanking regions was amplified by PCR from template plasmid pRS421 and primers pRS_5 and pRS_3 and used to co-transform a yeast strain (BY4741) with pJT3438, the *LEU2* marker gene of which harbors identical flanking regions, allowing for recombination-mediated marker exchange *in vivo*.

Table III

Primers Used in This Study

Names	Sequence (5′->3′)[a]
cdc10C266Sqc	ggatatcaaccagTCTgattttg
cdc10C266Sqc-r	caaaatcAGActggttgatatcc
cdc11C43F+NEW	cgaagtgtctacaacttgctgaccGAAcaaagtatttataaa
cdc11C43F-NEW	tttataaatactttgTTCggtcagcaagttgtagacacttcg
cdc11C137AC138A+NEW	ggacggcagagttcatGCTGCTctttacttaatcaaccc
cdc11C137AC138A-NEW	gggttgattaagtaaagAGCAGCatgaactctgccgtcc
cdc12C40Aqc	ccgtgatgttaGCTggtgagagcggattg
cdc12C40Aqc-r	caatccgctctcaccAGCtaacatcacgg
cdc12C278Sqc	cgattctcatTCTgatttccgtaagc
cdc12C278Sqc-r	gcttacggaaatcAGAatgagaatcg
cdc12C40VFw	cattcaccgtgatgttaGTTggtgagagcggattggg
cdc12C40VRv	cccaatccgctctcaccAACtaacatcacggtgaat
cdc12C278AFw	cgaaaacgattctcatGCTgatttccgtaagctcag
cdc12C278ARv	ctgagcttacggaaatcAGCatgagaatcgttttcg
cdc3C124Vqc+	ggattcagtttcaatctcctaGTTgtcggccctgatggtattgg
cdc3C124Vqc−	ccaataccatcagggccgacAACtaggagattgaaactgaatcc
cdc3C253Vqc+	cgataaaagaatccatgcaGTTctttatttcattgaacctacagg
cdc3C253Vqc−	cctgtaggttcaatgaaataaagAACtgcatggattcttttatcg
cdc3C279Vqc+	gcaatctgtatatgaaaaaGTTaacttgattcctgtcattgc
cdc3C279Vqc−	gcaatgacaggaatcaagttAACtttttcatatacagattgc
cdc3C124AFw	ggattcagtttcaatctcctaGCTgtcggccctgatggtattgg
cdc3C124ARv	ccaataccatcagggccgacAGCtaggagattgaaactgaatcc
cdc3C124SFw	gattcagtttcaatctcctaTCTgtcggccctgatggtattgg
cdc3C124SRv	ccaataccatcagggccgacAGAtaggagattgaaactgaatc
cdc3S407CFw	ctatacgaaaactacaggtctTGTaaattggcaaaacttggtatt
cdc3S407CRv	aataccaagttttgccaatttACAagacctgtagttttcgtatag
cdc10R298CFw	ttatgaagggttcagagcaTGTcaattaattgccttgaaag
cdc10R298CRv	ctttcaaggcaattaattgACAtgctctgaacccttcataa
cdc11E294CFw	gagatattatatgaaagatatagaaccTGTgcattatcaggtgaatctgtcgcg
cdc11E294CRv	cgcgacagattcacctgataatgcACAggttctatatctttcatataatatctc
cdc12L310CFw	attacgaaacatacagaagaTGTagacttgagggtcacgagaac
cdc12L310CRv	gttctcgtgaccctcaagtctACAtcttctgtatgtttcgtaat
cdc10seq2	ctgcaaacggaatgatag
cdc10seq3fw	gcttgggagcctattgtgaag
cdc10RS−	aacataagatatataatcaccaccattc
LA10re	ccatcgattggtggacgttgaatggcgttg
cdc11RS−	agaaagaagaaataagtgaggaagccaaaagcggactcacagattgtactgagagtgcac
cdc11FWD-new	tggctggtggttagcaacttt
cdc11delchisR	ttctaaccgtatttgctcctca

Names	Sequence (5′->3′)[a]
cdc12term_RS−	aaaccggtttatttttgtttttactgttcgattgtgttacagattgtactgagagtgcac
12GEXFW	attaggatcctgagtgctgccactgcgactgc
Duet12Xhoire	ccgactcgagtcattttaaatgggatttttttac
cdc12endRS+new	ttgcaagtaaaaaaatcccatttaaaatgactcgagtcggctgtgcggtatttcacaccg
12t-gfpre	attaggatccgtgtgtgacaatttccttgctgg
cdc3seq2	cttaatctccggttggtc
cdc3RS−	taatagtgtatgtttgaaatttttatatgtctttatttcgagattgtactgagagtgcac
cdc3genREV	gcgctctagatgtttgaaatttttatat
pRS_5	gtgtcggggctggcttaactatgcggcatcagagcagattgtactgagagtgcac
pRS_3	acaatttcctgatgcggtattttctccttacgcatctgtgcggtatttcacaccg

[a] Uppercase letters indicate nucleotides encoding Cys substitutions.

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Table IV

Growth and Morphology Characteristics of the Constructed Yeast Strains

	Colony growth			Cellular morphology			Septin localization		
Genotype (strain)	**26°C**	**30°C**	**37°C**	**26°C**	**30°C**	**37°C**	**26°C**	**30°C**	**37°C**
Wild-type (BY4741 [pJT2675])	+++	+++	+++	+++	+++	+++	+++	+++	+++
Wild-type (BY4742 [pJT2675])	+++	+++	+++	+++	+++	+++	+++	+++	+++
cdc10(C266S)-GFP (JTY4984)	++	++	+	+++	+++	+++	+++	+++	++
cdc10(C266S)-GFP cdc12(C40A C278S) (JTY4992)	++	++	+	+	+	+	++	++	+
cdc12(C40A C278S) (JTY4998)	+++	+++	+++	+	+	+	NA[a]		
cdc12(C40V C278A) (JTY5029)	+++	+++	+++	+	+	+	NA		
cdc12(C40V) (JTY5028)	+++	+++	+++	++	++	++	NA		
cdc12(C40A) (JTY4999)	+++	+++	+++	++	++	++	NA		
cdc12(C278S) (JTY5000)	+++	+++	+++	++	++	++	NA		
cdc3(C124V C253V C279V)	−	−	−	NA			NA		
cdc3(C124V)	−	−	−	NA			NA		
cdc3(C253V)	−	−	−	NA			NA		
cdc3(C279V)	−	−	−	NA			NA		
cdc3(C124S)	−	−	−	NA			NA		
cdc11(C43F C137A C138A) (JTY5001)	+++	+++	+++	+++	+++	+++	NA		
cdc11(C137A C138A) (JTY5001)	+++	+++	+++	+++	+++	+++	NA		
cdc11(C43F C137A C138A cdc12(C40A C278S) (JTY5018)	+	+	−	+	+	+	NA		
cdc11(C43F C137A C138A) cdc10(C266S)-GFP(JTY5019)	++	++	+	+++	+++	++	+++	+++	++
cdc10(C266S)-GFP cdc11(C43F C137A C138A) cdc12(C40A C278S) (JTY5017)	+	+	−	−	−	−	+	+	+
cdc3Δ cdc10(C266S)-GFP cdc11(C43F C137A C138A) cdc12(C40A C278S [***cdc3 (C124V C253V C279V)***] (JTY5043)	+++	+++	−	+	+	+	+	+	+

[a] NA: not applicable.