Higher-Order Septin Assembly Is Driven by GTP-Promoted Conformational Changes: Evidence From Unbiased Mutational Analysis in Saccharomyces cerevisiae

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ABSTRACT Septin proteins bind GTP and heterooligomerize into filaments with conserved functions across a wide range of eukaryotes. Most septins hydrolyze GTP, altering the oligomerization interfaces; yet mutations designed to abolish nucleotide binding or hydrolysis by yeast septins perturb function only at high temperatures. Here, we apply an unbiased mutational approach to this problem. Mutations causing defects at high temperature mapped exclusively to the oligomerization interface encompassing the GTP-binding pocket, or to the pocket itself. Strikingly, cold-sensitive defects arise when certain of these same mutations are coexpressed with a wild-type allele, suggestive of a novel mode of dominance involving incompatibility between mutant and wild-type molecules at the septin–septin interfaces that mediate filament polymerization. A different cold-sensitive mutant harbors a substitution in an unstudied but highly conserved region of the septin Cdc12. A homologous domain in the small GTPase Ran allosterically regulates GTP-binding domain conformations, pointing to a possible new functional domain in some septins. Finally, we identify a mutation in septin Cdc3 that restores the high-temperature assembly competence of a mutant allele of septin Cdc10, likely by adopting a conformation more compatible with nucleotide-free Cdc10. Taken together, our findings demonstrate that GTP binding and hydrolysis promote, but are not required for, one-time events—presumably oligomerization-associated conformational changes—during assembly of the building blocks of septin filaments. Restrictive temperatures impose conformational constraints on mutant septin proteins, preventing new assembly and in certain cases destabilizing existing assemblies. These insights from yeast relate directly to disease-causing mutations in human septins.

HETEROOLIGOMERIC septin assemblies control cytokinesis, microtubule-based vesicular transport, ciliogenesis, bacterial entry into host cells during infection, phagocytosis, chromosome alignment, and formation of spines in dendrites and collateral branches in axons, among other cellular processes (Hu et al. 2012; Mostowy and Cossart 2012; Ghossoub et al. 2013). In most of these cases, heterohexameric or -octameric septin complexes appear to polymerize into filaments capable of lateral “bundling” associations, frequently appearing as plasma-membrane-associated rings. Filamentous septin assemblies act as scaffolds and membrane diffusion barriers to modify membrane properties and restrict in space and time the localization of other proteins (Oh and Bi 2011).

In many organisms, distinct isoforms of particular septin subunits are expressed in a cell-type-specific manner and are often coexpressed in a given cell type. Isoforms occupy the same position within the heterooligomer and may be products of distinct genes or variants of a single gene diversified by alternative splicing or other mechanisms. Specific isoforms are able to target septin heterooligomers to specific cellular functions (García et al. 2011; Kim et al. 2011; Sellin et al. 2012), presumably by tailoring the structural properties of higher-order septin assemblies to the task at hand, and/or modifying the repertoire of nonseptin proteins.
and other factors (e.g., membrane lipids) with which the heterooligomers interact. In particular, the “terminal” subunit of septin heterooligomers, which mediates the ability of heterooligomers to polymerize into filaments, appears to be a common site of diversification (see the example of Shs1 in Figure 1A). Indeed, mutations in the terminal subunits are associated with human disease: SEPT9 in hereditary neuralgic amyotrophy (Kuhlenbäumer et al. 2005) and SEPT12 in male infertility (Kuo et al. 2012).

All septins studied to date bind GTP, but only some hydrolyze GTP to GDP, and significant rates of nucleotide exchange have not been observed for native septin heterooligomers (Vrabioiu et al. 2004; Farkasovsky et al. 2005). Indeed, within heterooligomers/filaments, the GTP-binding pockets are largely inaccessible to solvent, as a major oligomerization interface (called the “G interface”) buries the pockets (see Figure 1) and precludes hydrolysis/exchange-mediated dynamics that are observed in other cytoskeletal polymer systems. Nonetheless, GTP binding and hydrolysis clearly play important roles in the assembly of septin heterooligomers. As in other Ras-like small GTPases, conformational changes in the “switch” regions of septins accompany GTP hydrolysis (Sirajuddin et al. 2009). Rather than altering interactions with “effector” proteins, however, these changes appear to modify interactions with other septins across the G interface (Sirajuddin et al. 2009). GTP hydrolysis also alters the conformation of the “NC” interface by which G dimers associate with other G dimers to form rod-shaped protofilaments (Sirajuddin et al. 2009), suggestive of allosteric effects that could couple the nucleotide status in the pocket of one septin to interactions between other septins. Importantly, in each case of human infertility caused by septin mutation, the mutation affected a residue predicted to contact bound nucleotide and either blocked GTP binding or hydrolysis, leading to the complete failure to assemble a filamentous septin ring called the annulus (Kuo et al. 2012).

Sepin-encoding genes were first identified in an unbiased chemical mutagenesis screen for temperature-sensitive (Ts−)
yeast strains (Hartwell 1971). At high temperature, cells carrying mutant alleles of one of only four genes—CDC3, CDC10, CDC11, and CDC12—lack an array of filaments at the mother–bud neck (Longtine et al. 1996) that are thought to be composed of the septins themselves (Oh and Bi 2011). The amino acid changes in seven Ts– septin mutants isolated in unbiased screens have been identified: cdc10-1 (D182N) (McMurray et al. 2011b), cdc10-11 (G179D) (Cid et al. 1998), cdc11-1 and cdc11-7 (G29D) (Nagaraj et al. 2008), cdc11-6 (G32E) (Nagaraj et al. 2008), cdc12-1 (G247E) (Casamayor and Snyder 2003), and cdc12-6 (L391N E392STOP) (Nagaraj et al. 2008). Six of these substitutions affect residues that are predicted—based on homology and using the atomic structures of human septins (Sirajuddin et al. 2007; 2009; Macedo et al. 2013)—to lie within the GTP-binding pocket, either near or in direct contact with bound nucleotide. By contrast, cdc12-6 encodes a C-terminally truncated protein with a single substitution far from the pocket, eliminating a portion of a predicted coiled-coil-forming domain that is not conserved among all septins (Pan et al. 2007). Analysis of the effect of high cultivation temperature on the septin-ring-forming ability of mutant cells suggested a functional distinction between the GTP-binding-pocket mutants and the coiled-coil mutant: the pocket mutants were reportedly able to maintain a septin ring at high temperature, provided the ring had assembled at low temperature, whereas in coiled-coil-mutant cells even preformed rings disappeared at high temperature (Dobbeleare et al. 2003; Nagaraj et al. 2008). The steady-state levels of both types of mutant proteins are indistinguishable from wild type at any temperature (Nagaraj et al. 2008), and defects in the behavior of the pocket-mutant proteins can be detected even at permissive growth temperatures [as reduced incorporation of the mutant protein in heteromeric septin complexes (Nagaraj et al. 2008), and synthetic genetic phenotypes when combined with mutations in other genes (Longtine et al. 1996; Nagaraj et al. 2008)]. These findings suggested that the effect of temperature is not on the ability of the pocket-mutant proteins to bind nucleotide or to escape destruction. Instead, high temperature appeared to prevent pocket-mutant cells from assembling new septin complexes that were competent to form a filamentous ring, pointing to a structural role for GTP binding in septin–septin interactions (Nagaraj et al. 2008). Somewhat paradoxically, however, rings formed from complexes containing pocket-mutant proteins were thermostable, suggesting that any structural role of nucleotide is dispensable once the ring form is achieved. In coiled-coil-mutant (cdc12-6) cells, on the other hand, either the complexes themselves or their ability to polymerize into cortical filaments is fundamentally thermolabile.

As an unbiased approach to learn more about the requirements for yeast septin ring assembly and stability, we identified the amino acid changes in septin proteins responsible for the temperature sensitivity of an additional 25 Ts– yeast strains and five cold-sensitive (Cs–) strains. The locations of the affected residues and our characterization of ring defects in mutant cells identify septin dimerization at the G interface—rather than GTP binding per se—as a critical step in ring assembly. Our data suggest a novel mechanism of quality control of septin ring formation and point to an inability to acquire functional conformations as the primary effect of temperature in Ts– and Cs– septin mutants.

Materials and Methods

Strains, media, and genetic manipulations

All yeast strains are listed in Table 1 and were manipulated using standard techniques (Lundblad and Struhl 2001). Yeast cells were cultivated in liquid or on solid agar plates of rich (YPD, 1% yeast extract, 2% tryptone or peptone, 2% dextrose) or synthetic (Drop Out Mix Minus various ingredients, United States Biological) media, as appropriate to maintain plasmid selection. G-418 (Geneticin) was added to YPD to a final 200 μg/ml of the active drug. Hydroxyurea (HU) was dissolved in YPD at 0.2 M. Sporulation was induced in 1% potassium acetate, 0.05% glucose, 20 mg/liter leucine, 40 mg/liter uracil. Bacterial strains DH5alpha and XL1-Blue (Agilent) were used to propagate plasmids (Table 2), of which DNA was obtained as for yeast DNA (see below) but without glass bead lysis. Yeast transformation was performed using the Frozen-EZ Yeast Transformation Kit II (Zymo Research).

Preparation of yeast DNA, PCR, cloning, PFGE, and Array-Based Comparative Genomic Hybridization

For PCR, genomic or plasmid DNA from yeast was isolated by resuspending cells in 250 μl P1 (50 mM Tris-HCl pH 8.0, 10 mM EDTA, 100 μg/ml RNase A), adding 0.5-mm glass beads to displace an additional ~250 μl of volume and vortexing for 3 min prior to addition of P2 (200 mM NaOH, 1% SDS) and N3 (4.2 M Guanidinium-HCl, 0.9 M potassium acetate, pH 4.8). Lysates were clarified by centrifugation and applied to Zippy plasmid DNA miniprep columns (Zymo Research); then DNA was washed and eluted according to the manufacturer’s instructions. Plasmids were transformed to competent bacterial cells using the Mix & Go E. coli Transformation Kit (Zymo Research) or supercompetent XL1-Blue cells according to the manufacturer’s instructions. PCR was performed with various high-fidelity enzymes, typically Q5 (New England Biolabs), according to the manufacturer’s instructions. Primers and nucleotides were eliminated prior to dideoxy sequencing by treatment of the PCR reaction with Exol and FastAP (Fermentas), according to the manufacturer’s instructions. For those PCR products that were TOPO® cloned, pCR4Blunt-TOPO® (Life Technologies) was used according to the manufacturer’s instructions. PFGE and array-based comparative genomic hybridization (array-CGH), along with yeast DNA preparation for those techniques, were performed as described previously (Argueso et al. 2008).
Table 1 Yeast strains used in this study and the phenotypes of septin mutations

| Strain | Relevant genotype | Temperature-sensitive phenotype(s) | Septin region affected; predicted nucleotide state | Source/reference |
|--------|------------------|-----------------------------------|---------------------------------------------------|-----------------|
| LH10004 | A364A “cdc3-3” cdc3(G365R) | Recessive Ts: growth | “WG” motif of G interface; GTP | Hartwell (1971) |
| JPT2 | S288C “cdc3-6” cdc3(G365R) | Recessive Ts: growth | “WG” motif of G interface; GTP | Adams and Pringle (1984) |
| JPTA1506 | S288C cdc3(G365R) | Recessive Ts: growth | “WG” motif of G interface; GTP | J. Pringle |
| JPTA1509 | S288C cdc3(G365R) | Recessive Ts: growth | “WG” motif of G interface; GTP | J. Pringle |
| CBY04956 | BY4741 “cdc5-1” cdc3(G365R):kanMX | Recessive slow growth | “WG” motif of G interface; GTP | Li et al. (2011) |
| CBY07236 | BY4741 “cdc3-3” cdc3(G365R):kanMX | Recessive Ts: growth, ring assembly (pLP29) | “WG” motif of G interface; GTP | Li et al. (2011) |
| CBY06417 | BY4741 “cdc10-1” cdc10(D182N):kanMX | Recessive Ts: growth, ring stability (pLP29) | G4 motif; empty | Li et al. (2011) |
| CBY06420 | BY4741 “cdc10-2” cdc10(G100E):kanMX | Recessive Ts: growth, ring stability (pML43) | G3 motif; empty | Li et al. (2011) |
| LH310-2 | A364A “cdc3-3” cdc10(G39D) | Recessive Ts: growth | P-loop; guanosine? | Hartwell (1971) |
| CBY06421 | BY4741 “cdc10-4” cdc10(G100E V115A S317N):kanMX | Recessive Ts: growth | G3 motif; unknown | Li et al. (2011) |
| CBY06424 | BY4741 “cdc10-5” cdc10(P3S G44D):kanMX | Recessive Ts: growth, ring stability (pML43) | P-loop; guanosine? | Li et al. (2011) |
| JPT193 | S288C cdc10(D182N) | Recessive Ts: growth | G4 motif; empty | J. Pringle |
| VCY1 | leu2-3,112 trp1-1 ura3-52 his4 “cdc10-11” cdc10(G179D) | Recessive Ts: growth, ring stability (pML43) | G4 motif; empty | Cid et al. (1998) |
| CBY08756 | BY4741 “cdc11-1” cdc11(G32E J142T):kanMX | Recessive Ts: growth, ring stability (pML43) | P-loop; guanosine? | Li et al. (2011) |
| CBY06426 | BY4741 “cdc11-2” cdc11(G29D L368S):kanMX | Recessive Ts: growth, ring assembly (pLP29) | P-loop; guanosine? | Li et al. (2011) |
| CBY06427 | BY4741 “cdc11-3” cdc11(G29D):kanMX | Recessive Ts: growth, ring assembly (pLP29) | P-loop; guanosine? | Li et al. (2011) |
| CBY06525 | BY4741 “cdc11-4” cdc11(S31F S100P):kanMX | Recessive Ts: growth, ring assembly (pLP17) | P-loop; guanosine? | Li et al. (2011) |
| CBY06530 | BY4741 “cdc11-5” cdc11(G34D):kanMX | Recessive Ts: growth, ring assembly (pLP17) | P-loop; guanosine? | Li et al. (2011) |
| JPT194 | S288C “cdc11-6” cdc11(G32E) | Recessive Ts: growth | P-loop; guanosine? | Adams and Pringle (1984) |
| JPT9 | S288C “cdc11-7” cdc11(G29D) | Recessive Ts: growth | P-loop; guanosine? | J. Pringle |
| C17.01D | S288C cdc11(G29D)/CDC11 | Dominant Cs+ growth | P-loop; guanosine? | Moir et al. (1982) |
| N84.06D | S288C cdc11(G29D)/CDC11 | Dominant Cs+ growth | P-loop; guanosine? | Moir et al. (1982) |
| P44.08C | S288C cdc11(G32E)/CDC11 | Dominant Cs+ growth | P-loop; guanosine? | Moir et al. (1982) |
| Q26.15D | S288C cdc11(G32E)/CDC11 | Dominant Cs+ growth | P-loop; guanosine? | Moir et al. (1982) |
| K3534 | MATa ade2-1 ade3 trp1-1 can1-100 leu2-3,112 ura3 cdc3::his3 cts2a [YCpURA3 ADE3 PADV;CLN2] “cla10-3” bud4 | Normal growth | P-loop; guanosine? | Promoter; GDP | Cvrcková et al. (1995) |
| K3535 | MATa ade2-1 ade3 trp1-1 can1-100 leu2-3,112 ura3 cdc3::his3 cts2a [YCpURA3 ADE3 PADV;CLN2] “cla10-1” bud4 cdc12(R363K) | Cs+ growth | Ran-like C-terminal extension; GDP | Cvrcková et al. (1995) |
| K3536 | MATa ade2-1 ade3 trp1-1 can1-100 leu2-3,112 ura3 cdc3::his3 cts2a [YCpURA3 ADE3 PADV;CLN2] “cla10-4” bud4 | Normal growth | Promoter; GDP | Cvrcková et al. (1995) |
| K3538 | MATa ade2-1 ade3 trp1-1 can1-100 leu2-3,112 ura3 cdc3::his3 cts2a [YCpURA3 ADE3 PADV;CLN2] “cla10-2” bud4 cdc12(G247E)/CDC12 | Dominant Ts-, Cs+ growth | Nucleotide binding pocket; CTP | Cvrcková et al. (1995) |

(continued)
| Strain       | Relevant genotype | Temperature-sensitive phenotype(s) | Septin region affected; predicted nucleotide state | Source/reference |
|-------------|-------------------|-----------------------------------|---------------------------------------------------|-----------------|
| CBY05110    | BY4741 “cdc12-1”  | Recessive Ts− growth, dominant    | Nucleotide binding pocket; CTP                    | Li et al. (2011) |
|             | cdc12(G247E)::kanMX | Ts− morphology, ring assembly (pML43) |                                                   |                 |
| CBY05569    | BY4741 “cdc12-td” | Recessive Ts− growth, ring        | N-terminal fusion to temperature-sensitive       | Li et al. (2011) |
|             | Arg-DHFR<sup>b</sup>−CDC12::kanMX | stability (pML43) | degron; GDP |                 |
| JPT116      | S288C cdc12(G247E) | Ts− growth                       | Nucleotide binding pocket; CTP                    | J. Pringle      |
| JPTA1435    | S288C cdc12(G268R) | Ts− growth                       | “WG−” motif of G interface; GDP                  | J. Pringle      |
| JPTR122     | S288C cdc12(G247E) | Ts− growth                       | Nucleotide binding pocket; CTP                    | J. Pringle      |
| JPTR123     | S288C cdc12(G247E) | Ts− growth                       | Nucleotide binding pocket; CTP                    | J. Pringle      |
| BY4741      | MATα his3Δ1 leu2Δ0 ura3Δ0 met15Δ0 | Normal growth                   | N/A; N/A                                         | Brachmann et al. (1998) |
| BY4742      | MATα his3Δ1 leu2Δ0 ura3Δ0 lys2Δ0 | Normal growth                   | N/A; N/A                                         | Brachmann et al. (1998) |
| BY4743      | MATα his3Δ1/his3Δ1 leu2Δ0/leu2Δ0 ura3Δ0/ura3Δ0 met15Δ0/MET15 lys2Δ0/IYS2 | Normal growth                   | N/A; N/A                                         | Brachmann et al. (1998) |
| JTY4024     | BY4742 shs1Δ::kanMX | Cs− morphology                   | Protein absent                                   | Winzeler et al. (1999) |
| MMY0104     | BY4742 hoΔ::kanMX | Normal growth                    | N/A                                              | Winzeler et al. (1999) |
| MMY0127<sup>d</sup> | shs1Δ::kanMX cdc11(G34D)::kanMX [pML111] | Ts− growth, ring stability (pML111) | Shs1: absent. Cdc11: P-loop; guanosine? | This study      |
| MMY0110<sup>g</sup> | JPT193 cdc3(D210G) | Normal growth                    | Cdc10: G4 motif; empty. Cdc3: switch II, GTP     | This study      |
| MMY0128<sup>f</sup> | cdc10(D182N) | Ts− growth                       | G4 motif; empty                                  | This study      |
| MMY0129<sup>f</sup> | CDC10 CDC3 | Normal growth                    | N/A; N/A                                         | This study      |
| MMY0130<sup>f</sup> | cdc3(D210G) cdc10(D182N) | Normal growth                    | Cdc10: G4 motif; empty. Cdc3: switch II, GTP     | This study      |
| MMY013<sup>f</sup> | cdc3(D210G) | Slow growth at all temps         | Switch II, GTP                                   | This study      |

<sup>a</sup> Septin mutations presumed to be responsible for the temperature-sensitive phenotype(s) are shown in boldface type.

<sup>b</sup> Where the distinction between failure to assemble (“ring assembly”) or failure to maintain (“ring stability”) a septin ring at high temperature has been made experimentally, the fluorescently tagged septin plasmid used is given in parentheses.

<sup>c</sup> N/A, not applicable. “?” indicates speculation without supporting evidence.

<sup>d</sup> Spore from cross of diploid strain made by mating JTY4024 and CBY06430 and introducing pML111 by transformation.

<sup>e</sup> Spontaneous suppressor of Ts− phenotype of strain JPT193.

<sup>f</sup> Spore from cross of diploid strain made by mating BY4741 and CBY06430 and introducing pML111 by transformation.

<sup>g</sup> Spore from cross of diploid strain made by mating BY4741 and CBY06430 and introducing pML111 by transformation.
to water, spotted on agarose pads, and visualized by micros-
250-msec exposures at 100% intensity. Imaging of Cdc10
paused in 1 ml of 0.5
then pelleted, washed three times with water, and resus-
dents in small selective liquid cultures and then divided in half, pel-
cells were grown to mid-log phase at room temperature in
DAPI
in the dark. DAPI-stained nuclei were visualized with the

Results
We collected Ts− mutants that were identified in chemical mu-
tagenesis screens and determined by linkage and/or comple-
mentation to carry septin mutations (Table 1). These included
six cdc3, six cdc10, seven cdc11 (for cdc11-1, we obtained a dif-
fent result than previously published; Nagaraj et al. 2008),
and five cdc12 mutants (Figure 1B). Alleles of CDC11 had also
been found in similar screens for cold-sensitive (Cs−) mutants
(Moir et al. 1982; Healy et al. 1991), so we analyzed four of
these. Finally, as intended negative controls, we obtained four
dla10 mutants derived from a temperature-independent screen
for mutants that are synthetically lethal with depletion of G1
cyclins (Cvrcková and Nasmyth 1993) and were determined by
complementation to be alleles of CDC12 (Cvrcková et al. 1995).

Table 2  Plasmids used in this work

| Plasmid                  | Relevant properties                     | Source/reference         |
|-------------------------|----------------------------------------|--------------------------|
| pCdc10-1-GFP            | CEN URA3 cdc10(D182N)-GFP              | McMurray et al. (2011b)  |
| pFA6a-KanMX4            | kanMX4                                 | Wach et al. (1998)       |
| pML43                   | CEN LEU2 his3MX CDC11-YFP              | Nagaraj et al. (2008)    |
| pML111                  | CEN LEU2 his3MX CDC10-YFP              | Nagaraj et al. (2008)    |
| pSB1                    | CEN URA3 CDC11                         | Versele et al. (2004)    |
| pLP17                   | CEN LEU2 CDC12-GFP                     | Lippincott and Li (1998) |
| pLP29                   | CEN HIS3 CDC12-GFP                     | Lippincott and Li (1998) |
| pCR4Blunt-TOPO          | Topoisomerase-coupled cloning vector    | Life Technologies       |
| pTOPO-JPT116a           | pCR4Blunt-TOPO cdc12(G247E)            | This work                |
| pTOPO-JPTR122a          | pCR4Blunt-TOPO cdc12(G247E)            | This work                |
| pTOPO-JPTR123a          | pCR4Blunt-TOPO cdc12(G247E)            | This work                |
| YCpK-Cdc10-1-GFPp       | CEN kanMX cdc10-1-GFP                  | This work                |

* A central portion of the CDC12 coding sequence from strains JPT116, JPTR122, or JPTR123 was amplified with PFU and cloned into pCR4Blunt-TOPO*

* pCdc10-1-GFP was cotransformed into strain BY4742 with a PCR product amplified from template pFA6a-KanMX4 that contained the kanMX4 cassette flanked by sequences targeting it for replacement via homologous recombination of the URA3 marker in pCdc10-1-GFP.

Microscopy

All images were captured with an EVOSfl (Advanced Microscopy Group) all-in-one microscope equipped with an Olympus 60× oil immersion objective and GFP, YFP, and DAPI filters. For assembly-versus-stability experiments, yeast cells were grown to mid-log phase at room temperature in small selective liquid cultures and then divided in half, pelleted, and resuspended in 100 μl YPD or 100 μl YPD + HU. Cultures were transferred to thin-wall PCR tubes and incubated in a thermal cycler with the following program: 4 hr at 23°C, 2 hr at 37°C, and hold at 4°C. Cells were then transferred to water, spotted on agarose pads, and visualized by microscopy with the GFP or YFP filter, as appropriate, and 60- or 250-msec exposures at 100% intensity. Imaging of Cdc10 (D182N)-GFP was performed in a similar way with cells from a mid-log YPD + G418 culture. For DAPI staining, cells were pelleted, resuspended in 1 ml 70% ethanol for 5 min, then pelleted, washed three times with water, and resuspended in 1 ml of 0.5 μg/ml DAPI before a 1-hr incubation in the dark. DAPI-stained nuclei were visualized with the DAPI filter using a 60-msec exposure at 100% intensity.

Cold-sensitive cdc11 mutants harbor two alleles, one wild-type and one mutant allele that alone makes cells heat sensitive

Our analysis of the four Cs− cdc11 mutants generated surprising results. In each, one or more positions in CDC11 appeared as “mixed” peaks with two distinct nucleotides
clearly represented (Figure 2A), indicative of two alleles of \textit{CDC11} in the same purportedly haploid strain (Moir et al. 1982). Two strains (C17.01D and N84.06D) had the same six mixed positions, of which only one was a coding change (G29D), and the other two strains (P44.08C and Q26.15D) had the same single mixed position, causing the coding change G32E. To confirm the presence of two \textit{CDC11} alleles in C17.01D, we exploited a restriction site introduced by one of the silent mutations and digested a PCR product amplified from one such strain, yielding the expected pattern of one uncut species and one singly cut species (Figure S2A). Remarkably, the coding changes are identical to P-loop anticodon exceptions: loci that in the reference strain harbored deletion alleles of auxotrophic markers (\textit{ura3\Delta0} and \textit{lys2\Delta0}) were overrepresented relative to the \textit{URA3} \textit{LYS2} Cs\textsuperscript{−} strains, and loci that frequently undergo spontaneous changes in copy number (\textit{CUP1}, \textit{Ty1} transposable elements) were present at variable levels in some strains (Figure 2C and data not shown). For many of the former instances, the copy number ratio was \(~0.5\) relative to the rest of the genome (data not shown), demonstrating that, for each Cs\textsuperscript{−} strain, most of the genome, including \textit{CDC11}, is present at two copies per cell.

We hypothesized that the two alleles arose from a local duplication of the \textit{CDC11} locus and amplified and sequencing nearby genes to identify those without mixed peaks that would define the boundaries of the duplication. To our surprise, in addition to mixed peaks in the genes that flank \textit{CDC11} on chromosome (Chr) X, \textit{HOC1} and \textit{MIR1}, we detected two alleles of \textit{HAM1} (Figure S2B), \(~7\) kb away, suggesting the duplication might be large enough to alter the mobility of Chr X in pulsed-field gel electrophoresis (PFGE). This analysis revealed a range of apparent karyotypes in the Cs\textsuperscript{−} mutants: some appeared normal, whereas strain N84.06D harbored at least one extra, missized copy of nearly every chromosome (Figure S2C).

Ultimately, we used array-CGH to quantify the copy number of nearly every protein-coding locus in the genome in the four \textit{cdc11} Cs\textsuperscript{−} strains. Relative to a reference strain (BY4741), \textit{CDC11} (Figure 2C) and the vast majority of other loci were present at the same copy number, with a few anticipated exceptions: loci that in the reference strain harbored deletion alleles of auxotrophic markers (\textit{ura3\Delta0} and \textit{lys2\Delta0}) were overrepresented relative to the \textit{URA3} \textit{LYS2} Cs\textsuperscript{−} strains, and loci that frequently undergo spontaneous changes in copy number (\textit{CUP1}, \textit{Ty1} transposable elements) were present at variable levels in some strains (Figure 2C and data not shown). For many of the former instances, the copy number ratio was \(~0.5\) relative to the rest of the genome (data not shown), demonstrating that, for each Cs\textsuperscript{−} strain, most of the genome, including \textit{CDC11}, is present at two copies per cell.

Two other copy number variations were observed in strain C17.01D. A portion of the \textit{SAK1} gene was amplified (Figure S2D), and there was a segmental deletion between...
the ALD3 and ALD2 genes (data not shown). Sak1 is a member of the SNF1/AMPK family of protein kinases, whose other members in yeast are Tos3 and Elm1. Elm1 regulates septin function in poorly understood ways, likely by activating septin kinases (Asano et al. 2006). Elm1, Sak1, and Tos3 share overlapping function in the phosphorylation of Snf1 (Hong et al. 2003), but whether heterozygous diploid cells are generally unable to septin function despite the

presence of wild-type CDC11. This condition appeared to be unstable, because many cold-insensitive derivatives spontaneously appeared when the Cs– strains were plated to 14°C (data not shown). However, amplification and sequencing of CDC11 from these derivatives generated the same mixed peaks at the same positions (Figure 2D). We suspected that cells carrying both alleles might spontaneously lose one or the other, in either case generating a monoallelic Cs (cdc11) clone capable of 14°C growth. If this “sorting” process occurred after plating, the resulting colonies might each be a mix of the two clonal genotypes. To test this hypothesis, we passaged several “revertant” strains at 37°C, which should eliminate Ts–/cdc11(C292D) or cdc11(C32E) cells and thereby “purify” the population. Indeed, sequencing of the CDC11 gene from one of these populations revealed that only the wild-type allele remained (Figure 2D). By contrast, passaging at 37°C without prior selection at 14°C failed to eliminate either allele (data not shown), confirming that the diallelic Cs– clones are not Ts– and that at high temperatures the mutant allele does not interfere with septin function. We conclude that the presence of two CDC11 alleles—one wild type and one encoding a P-loop mutant—in the same cell is necessary but not sufficient to prevent septin function in the cold.

We realized that while our PFGE and array-CGH data demonstrate that the Cs– cdc11 strains have two copies per clone of most genes, these need not reside in the same nucleus: heterokaryons (cells with genetically distinct haploid nuclei sharing the same cytoplasm) would also generate the experimental results we observed. Indeed, DAPI staining of DNA in the C17.01D Cs– strain and examination by light
and fluorescence microscopy revealed that even at the permissive temperature of 22°C, most cells were linked together in branched chains of elongated cells with multiple nuclei per chain, similar to the terminal arrest phenotype at 14°C (Figure 2E). Thus, C17.01D may grow as a kind of coenocytic mycelium, in which nuclei with distinct genotypes could conceivably propagate relatively independently. The high frequency of revertants and the efficient allele sorting we observed are consistent with the mitotic instability of heterokaryons generated using a karyogamy mutant (Conde and Fink 1976).

A cold-sensitive cdc12 mutant identifies an uncharacterized, highly conserved domain that may allosterically influence the G interface

In the Cs–cla10-1 strain K3538, we discovered the mutation Cdc12 R363K, a highly conservative substitution within a region—between the “septin unique element” (Versele and Thorner 2005) and the hydrophobic heptad repeats predicted to form coiled coils (Figure 3A)—to which no structure or function has yet been assigned. To confirm that the R363K mutation was responsible for the cla10 and Cs– phenotypes, we obtained two independent Cs+ derivatives of K3538. Each had reverted to Arg at position 363, become auxotrophic for adenine, and formed white colonies (data not shown), indicating that they were now Cla10, and able to tolerate the depletion of G1 cyclins caused by loss of the ADE3 CLN3 plasmid (Cvrcková and Nasmyth 1993).

Among Cdc12 homologs in other fungal species, Arg 363 and the sequences immediately surrounding it are as highly conserved as the major GT-Pase motifs (Figure 3A). We further noticed that Gsp1 and Gsp2, paralogous relatives of the small GT-Pase Ran (Belhumeur et al. 1993), are the nonseptin Saccharomyces cerevisiae proteins with highest homology to Cdc12 (WU-BLAST2 P = 0.0025 and 0.0026, respectively), and that this homology is restricted to two motifs, the G1 box/P-loop and the portion of the Cdc12 C-terminal extension (CTE) that includes Arg 363 (Figure 3B). The homologous CTE in Ran (residues 173–216; Figure 3C) adopts distinct conformations in the GT-P- and GDP-bound states (Vetter et al. 1999). Deletion of the CTE creates a constitutively active Ran by altering the conformations of the switch I and II regions of Ran(GDP) to mimic the GTP-bound state (Nilsson et al. 2002). If the corresponding region in the Cdc12 CTE acts in a similar way to allosterically modify conformations of the Cdc12 switch regions, then the restrictive temperature might “lock” the R363K mutant Cdc12 into a mimic of the GTP-bound state that is either incompatible with G heterodimerization or interacts with Cdc11 to render it unable to NC dimerize (see Discussion).

Cdc12(G247E) acts dominantly to interfere with septin function

Strikingly reminiscent of our results with the Cs–cdc11 mutants, we detected multiple alleles of CDC12 in the moderately Ts– and Cs– strain K3538, one wild-type and one mutant allele found previously in several Ts– strains, cdc12(G247E) (Figure 4A, Table 1). Interestingly, this same mutation was first isolated (as cdc12-1) in the original cdc Ts– screen by Hartwell et al. (1973) and is the only such mutant that was not strictly recessive, instead described as behaving in a semidominant manner in “some” (unidentified) strains. The steady-state level of total Cdc12 relative to the metabolic enzyme glucose-6-phosphate dehydrogenase (Zwf1) was at least twofold higher in the K3538 (cla10-2) strain than in the other three cla10 strains (Figure S1B), suggesting that K3538 cells carry two copies of CDC12 but one of most other genomic loci. Furthermore, K3538 cells cultivated at 30°C appeared to be mononucleate (Figure 4B). We found that a heterozygous cdc12(G247E)/CDC12 diploid strain of the BY4743 background created by crossing a cdc12(G247E) haploid with a wild-type haploid proliferated normally at 37°C but exhibited a high proportion of cells (~25%) with morphological defects (Figure 4C). Thus, cdc12 (G247E) is unique among the G interface mutations described here (Table 1) or elsewhere (Nagaraj et al. 2008) in its ability to interfere in mononucleate cells with septin function in the presence of a wild-type allele of the same septin.

Septin-subunit-specific effects on the thermostability of septin rings

Others have examined the defects of certain Ts– septin mutants at high temperatures and found that GTP-binding-defective mutant cells were unable to assemble new rings, but preexisting rings were relatively stable (Dobbelaele et al. 2003; Nagaraj et al. 2008), consistent with bound GTP/GDP playing a structural role in new assembly that is less important in the thermal stability of the final product. We extended this analysis to unbiased mutants. S-phase arrest using HU allows rings assembled at low temperature to be tested for stability following a temperature upshift. Mutants clearly fell into two categories. Those in which
rings disappeared from most cells in both conditions were classified as “stability” mutants, whereas those in which rings were absent in the majority of cells without HU but visible in HU-arrested cells were classified as “assembly” mutants. Representative images for particular mutants are shown in Figure 5, and results for the rest are summarized in Table 1.

The cdc3(G365R) and all five cdc11 Ts− mutants tested were determined to be assembly mutants (Figure 5, Table 1, and data not shown), suggesting that initial G dimerization by the mutant molecules is inhibited by elevated temperatures, but the presence of mutant Cdc3 or Cdc11 subunits within the filamentous ring does not render it particularly thermostable. In addition to cdc12(G247E), we tested a temperature degron-tagged version of Cdc12 encoded by cdc12-td (Li et al. 2011). As expected from others’ results in a different strain background (Dobbelare et al. 2003), cdc12(G247E) was an assembly mutant (data not shown). cdc12-td was a stability mutant (data not shown), indicating that even when incorporated into the ring, Cdc12 molecules can be extracted and degraded by the proteasome, leading to disassembly of the entire structure. Unexpected was the observation that all five cdc10 mutants tested were stability mutants (Figure 5, Table 1, and data not shown), even those with identical substitutions in positions equivalent to cdc11 assembly mutants [e.g., Cdc10(G44D) and Cdc11(G34D), see Figure 1B]. These findings demonstrate that septin rings with mutant Cdc10 subunits are unstable at high temperature, in contrast with earlier studies in which a different, directed P-loop mutant of Cdc10 [cdc10(G42V)] mutant was deemed an assembly mutant (Nagaraj et al. 2008). However, after 90 min at 37°C the fraction of HU-arrested cdc10(G42V) cells with rings was the lowest of any nucleotide-binding-pocket mutant tested in that study (Nagaraj et al. 2008). We held HU-arrested cdc10 cells at 37°C for 120 min, which likely allowed us greater sensitivity in detecting ring stability effects. Moreover, the cdc10(G42V) mutant was only mildly Ts− (normal growth at 34°C) (Nagaraj et al. 2008), whereas the cdc10 mutants we examined grew poorly at 30°C and above (data not shown), suggesting that the directed G42V substitution was less effective at inhibiting nucleotide binding than unbiased mutants selected on the basis of their temperature sensitivity. We consider it unlikely that a given substitution in the nucleotide-binding pocket has a more severe effect on nucleotide binding by Cdc10 than by Cdc11. Instead, we wondered whether the septin ring might be better able to tolerate mutant Cdc11 subunits because there is another septin, Shs1, able to bind nucleotide and occupy the same position as Cdc11 during septin heteroocytamers (Figure 1A), potentially providing a stabilizing effect. To test this hypothesis, we deleted SHS1 in cdc11(G34D) cells and tested ring stability in the double-mutant cells. Indeed, septins rings, formed at low temperature in cdc11(G34D) shs1Δ cells, disappeared at 37°C despite S-phase arrest with HU (Table 1).

G dimerization, not nucleotide binding, is required for new ring assembly and ring stability at high temperatures

Bound nucleotide provides additional points of contact between two septins across a G dimer interface. At the same time, a G dimer interface provides additional points of contact to stabilize nucleotide binding. We set out to determine whether bound nucleotide is essential at high temperatures for the assembly of complexes competent for new septin ring formation at high temperatures, or, alternatively, whether nucleotide merely promotes the formation of G heterodimers that are essential for higher-order assembly. Pringle et al. (1986) noted decades ago that the most common spontaneous suppressors of Ts− mutations in Cdc10 are mutations in Cdc3, and vice versa. We
hypothesized that at high temperatures mutants of Cdc10 adopt conformations that are incompatible in association with wild-type Cdc3, but certain substitutions might render Cdc3 capable of “rescuing” the Cdc10 mutants by interacting with them, thereby bypassing the apparent requirement for nucleotide in high-temperature G heterodimerization.

The D182N substitution in Cdc10 that we identified in two independent cdc10 Ts− strains (Figure 1A, Table 1) very likely prevents GTP binding by Cdc10, as does the identical substitution in the corresponding position in human septin SEPT12 (Kuo et al. 2012). Cells of the cdc10(D182N) strain JPT193 were plated to 37°C and a spontaneous suppressor of the Ts− phenotype was isolated as a single colony after 3 days. The CDC10 and CDC3 coding regions were then amplified and sequenced. Consistent with our hypothesis, the original D182N mutation in CDC10 persisted, and we found a single substitution in Cdc3, D210G, altering an absolutely conserved residue within the G3 loop/switch II region of the G dimer interface (Sirajuddin et al. 2009) (Figure 1B). Directed mutagenesis of Cdc3 Asp210 to Ala makes cells unable to grow at high temperature and grow slowly at moderate temperatures (Sirajuddin et al. 2009). In a CDC10 background, cdc3(D210G) cells are not Ts− but also have a minor growth defect at all temperatures (Figure 6A), representing a rare example of reciprocal suppression: two mutations that alone cause clear phenotypes are able to rescue each other and restore normal growth when combined in the same strain.

Importantly, cdc3(D210G) does not simply allow cells to assemble polymerization-competent heterooligomers without Cdc10, as does the substitution G261V (McMurray et al. 2011a), because GFP-tagged Cdc10(D182N) was efficiently incorporated into the filamentous septin ring in cdc10(D182N) cdc3(D210G) cells (Figure 6C). We further find it exceedingly unlikely that the cdc3(D210G) mutation restores the ability of Cdc10(D182N) to bind and hydrolyze GTP. Accordingly, because septin rings are thermostable in cdc10(D182N) cdc3(D210G) cells, the ring instability observed in cdc10(D182N) CDC3 cells (Table 1) cannot be attributed to a structural requirement for GDP in the Cdc10 pocket. Instead, even when formed at low temperature, the Cdc3•GTP•Cdc10•empty G heterodimer and/or the Cdc10•empty•Cdc10•empty NC homodimer interfaces are probably unstable at high temperatures.

Discussion

Assembly of septin heterooctamers in discrete, semiordered steps

Certain of the Ts− mutants we analyzed were defective at high temperature specifically in the assembly of new rings. That rings in such assembly mutants are thermostable suggests that there is a discrete assembly step for which the act of nucleotide binding and/or G dimerization is important. Once this step has been accomplished, G dimers and all higher-order assemblies (octamers, filaments, rings) are stable.

A compelling candidate for this step is NC homodimerization: mammalian septins that cannot G dimerize are unable to NC homodimerize in vivo (Kim et al. 2012), but NC homodimerization mutants form normal G dimers (McMurray et al. 2011a; Kim et al. 2012). In vitro, when the G interface of human SEPT2 is altered by preventing GTP hydrolysis, the NC interface adopts a nonnative conformation incompatible with homodimerization (Sirajuddin et al. 2009). Thus, perturbing GTP binding by the septins that form NC homodimers—as in cdc10(G44D) and cdc11(G34D) shs1Δ stability mutants (Table 1)—may destabilize septin assemblies because in the context of a G dimer formed at the permissive temperature, these mutant septins adopt quasinative conformations in which NC homodimers cannot tolerate subsequent shifts to high temperatures. By contrast, NC heterodimers appear not to require prior G dimerization at any temperature, because immunoprecipitation experiments at the restrictive
temperature in yeast cells carrying Ts– alleles demonstrate that the Cdc3–Cdc12 NC heterodimer forms efficiently in cdc10 mutants wherein the Cdc10–Cdc3 G dimer fails to assemble, and in cdc11 or cdc12 mutants wherein the Cdc11–Cdc12 G dimer fails to assemble (Nagaraj et al. 2008). This view predicts a semidemerged assembly: nucleotide-facilitated formation of G heterodimers (Cdc3–Cdc10, Cdc11/Shs1–Cdc12) and nucleotide-independent Cdc3–Cdc12 NC heterodimerization occur simultaneously, followed by NC-homodimer-mediated joining of the resulting Cdc12–Cdc11–Cdc3–Cdc10 heterotramers into heterooolamers and ultimately into filaments (Figure 7A).

**Nucleotide binding promotes septin heterooligomerization via conformational changes in the subunit–subunit interfaces**

The majority of the substitutions we discovered in Ts– and Cs– septin mutants insert bulky and/or highly charged sidechains (Arg, Asp, Glu, and Phe) in place of small, uncharged ones (Gly and Ser) whose equivalents in septins of known structure are in close proximity to bound nucleotide (Figure 1, B and C). Steric and/or electrostatic interference with occupancy of the pocket by GTP/GDP is likely the most important consequence of such mutations, rather than loss of individual contacts with bound nucleotide: a bulky, charged G3 box substitution (G100E) caused Ts– growth in two cdc10 strains (Figure 1, A and B), whereas in earlier studies substituting the equivalent Gly in Cdc11 or Cdc12 to Ala had no phenotypic effect (Nagaraj et al. 2008). However, it is difficult to determine exactly to what extent these mutations prevent GTP binding or hydrolysis in vivo, since appropriate biochemical experiments are challenging, if not impossible.

Structural modeling predicts that Cdc10(D182N) and Cdc12(G247E) should render the pocket entirely incompatible with a guanosine base (C. Musselman, unpublished observations), but bulky substitutions in the P-loop—where contacts with the phosphates are made—might only restrict binding to certain guanosine phosphoforms. In support of this idea, we found a strong synthetic genetic interaction between the P-loop mutant cdc11(G29D) and cdc12(E188L), a directed mutation of a residue in Cdc12 predicted based on homology to human septins (Sirajuddin et al. 2009) to contact the guanosine base of the nucleotide bound in the pocket of Cdc11 (C. R. Johnson and M. A. McMurray, unpublished results). This Cdc12 mutation should have had little effect if the pocket of Cdc11(G29D) is nucleotide-free. Zent and Wittinghofer (2013) recently reported that human SEPT7 can bind nonphosphorylated guanosine, which favors a nonnative conformation of the G interface that is competent for assembly of a mixed dimer with SEPT7 bound to a nonhydrolyzable GTP analog (GppNHp). We speculate that at least some of the P-loop mutants of Cdc10 and Cdc11 bind guanosine or GMP in vivo, and the others prevent nucleotide binding altogether, in either case, biasing the mutant proteins toward nonnative conformations at high temperatures (Figure 7B).

Alternatively, considering that the chemical mutagens with which most of these mutant strains were created cause specific nucleobase alterations (e.g., G→A by EMS), certain septin proteins could be predisposed to acquire Ts– mutations if the codons representing critical residues are easily mutated to nonconservative amino acid substitutions (e.g., GGA→AGA and Gly→Arg). However, the specificity of P-loop mutations for Cdc10 and Cdc11 does not reflect biased codon usage at the relevant positions, because CDC3 and CDC12 use equivalent or identical codons for these highly conserved residues (data not shown). Instead, the pockets of Cdc3 and Cdc12 are apparently better able to tolerate even drastic single-residue substitutions without making cells Ts–, consistent with others’ observations using directed mutations of residues predicted to contact GTP [e.g., Cdc3(K132E) (Nagaraj et al. 2008) or Cdc3(D128A) (Sirajuddin et al. 2009)]. Indeed, the only single mutations in the pockets of Cdc3 or Cdc12 that cause strong growth defects are a directed substitution intended to block GTP hydrolysis by Cdc12—T75A, which kills cells even at moderate temperatures (Sirajuddin et al. 2009)—and Cdc12 G247E, which we identified in three Ts– strains (Figure 1B, Table 1). Interestingly, structural modeling suggests that the pocket of Cdc12 (G247E) could accommodate the pyrimidine triphosphate CTP (C. Musselman, unpublished observations). We propose that GTP binding per se by Cdc12 is not critical for its function in vivo. Instead, the Cdc12 pocket must not be occupied by a nucleoside triphosphate, i.e., GTP or CTP. Specifically, in Cdc12•GTP the conformations of the switch regions are incompatible with G dimerization with Cdc11. Cdc12•CTP adopts at all temperatures quasinative conformations that are competent for G dimerization, but in this G dimer neither septin is normal, just as in the dimer with SEPT7•GppNHp the SEPT7•(guanosine) is nonnative (Zent and Wittinghofer 2013). Indeed, in this scenario the nucleotide state of wild-type Cdc11 may also be altered (Figure 7C). The manifestations of these abnormalities depend on temperature and whether wild-type Cdc12•GDP is also present: at moderate and low temperatures, Cdc11 in heteroolamers containing Cdc12•CTP is able to mediate filament polymerization via NC homodimerization, but at high temperatures its NC interface is unable to homodimerize. When heteroolamers containing Cdc12•GDP are also present, native Cdc11 can interact with the nonnative Cdc11 molecules in Cdc12•CTP-containing heteroolamers, but only at moderate temperatures: in the cold, the two forms of Cdc11 are incompatible (Figure 7C). This model explains the Ts– phenotype of cdc12 (G247E) cells and the Ts– and Cs– phenotypes of the cdc12 (G247E)/CDC12 strain K3538 (Table 1). Cold-induced NC interface incompatibility between native and nonnative Cdc11 molecules also explains the Cs– phenotype of the four strains with one wild-type and one P-loop-mutant CDC11 allele (Figure 7D).

This scenario is strikingly similar to the apparent cause of certain cases of male infertility in humans: either of two
GTP-binding-pocket mutations in SEPT12, the septin subunit thought to occupy, like Cdc11, the terminal position in testis-specific septin octamers (Figure 1A) acts dominantly to block assembly of the annulus (Kuo et al. 2012). The T89M substitution, which eliminates the Thr predicted to catalyze GTP hydrolysis (Sirajuddin et al. 2009), allows

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**Figure 7** Models for conformational changes accompanying higher-order septin assembly and the effects of mutations and temperature. Nucleotide binding and hydrolysis states are speculative, as are the indicated conformational changes influencing septin–septin interactions. GTP is represented by three adjacent stars and GDP by two; CTP, three hexagons. G—, guanosine. Dashed box indicates incompatible/desibilized interface. (A) Stepwise assembly events in yeast septin heterooolter assembly. (B) P-loop-mutant Cdc11 molecules bind unphosphorylated guanosine, favoring at high temperatures a conformation of the G interface unable to dimerize with Cdc12. (C) Cells expressing both cdc12 (G247E) and CDC12. Speculative effects on GTP hydrolysis by Cdc11 are included. (D) As in B, but in the cold and the presence of wild-type (GDP-bound) Cdc11. (E) cdc12(R363K) cells cultivated in the cold. (F) As illustrated, in cdc3(D210G) CDC10+, CDC3+, cdc10(D182N), or cdc3(D210G) cdc10(D182N) cells cultivated at high temperatures.
SEPT12 to bind but not hydrolyze GTP (Kuo et al. 2012). SEPT12(D197N) prevents GTP binding altogether (Kuo et al. 2012) and is identical to the D182N substitution we identified in two cdc10 Ts mutant strains (Figure 1, B and C, Table 1). Following the rationale of temperature-dependent NC interface incompatibility between heterooctamers with terminal subunits in distinct conformations, we can easily imagine how, once incorporated into heterooctamers, SEPT12•GTP and nucleotide-free SEPT12 could block annulus assembly by wild-type proteins during spermatogenesis in the testis, where the temperature is ∼4° colder than the rest of the body. In a different GTP-binding protein, the α-subunit of the G protein-coupled receptor Gs, a mutation affecting a residue that also directly contacts bound nucleotide confers a testis-specific defect because the mutant protein is stable at 33° but degraded at 37°, suggestive of temperature-dependent conformational changes (Nakamoto et al. 1996). Similarly, the cold environment of the testis could lock the mutant sepsins into a filament-capping form.

Not all the Ts and Cs yeast mutations would be expected to cause defects in nucleotide binding per se. The cdc12(R363K) Cs– mutant altered a region of Cdc12 with homology to part of the GTPase Ran that controls the conformations of the switch regions (Nilsson et al. 2002). If in the cold Cdc12(R363K)•GDP mimics Cdc12•GTP, it could either destabilize the G interface with Cdc11 or induce in Cdc11 a conformation incompatible with NC homodimerization (Figure 7E). Additionally, one cdc12 and all six cdc3 Ts– strains harbored the same substitution in the same position (Cdc12 G268R; Cdc3 G365R), affecting a residue immediately adjacent to a Trp that makes a key G dimer contact outside the pocket (Sirajuddin et al. 2007; 2009; McMurray et al. 2011a; Kim et al. 2012) (Figure 1D). Phylogenetic analysis performed without structural knowledge identified this “Sep4” motif (WG) as being conserved in 92% of all sepsins (Pan et al. 2007). Among the other 8% is Cdc10, in which Ser (S256) is found instead of Gly (Figure 1C) and is the site of phosphorylation by the kinase Clu4 (Versele and Thoner 2004). Clu4 appears to promote sepsin ring assembly in parallel with nucleotide binding (Versele and Thoner 2004; Nagaraj et al. 2008), presumably by stabilizing sepsin–sepsin interactions like Cdc3–Cdc10. Thus, the substitutions to Arg we identified in the mutants could destabilize the Cdc3–Cdc10 or Cdc12–Cdc11 G interface at high temperatures while still allowing both protomers to interact normally with the nucleotides in their pockets.

Quality control of higher-order sepsin assembly: a “locals-only” competition?

Our models in which mutant sepsins act dominantly to interfere with sepsin function do not account for a “quality control” mechanism identified by others in which a wild-type sepsin “outcompetes” various nucleotide-binding-pocket mutant alleles for incorporation into sepsin rings at temperatures permissive for function of the mutants (Cid et al. 1998; Nagaraj et al. 2008). Cdc42 was proposed to preferentially recruit nucleotide-replete octamers to the site of sepsin ring assembly (Nagaraj et al. 2008). The dominance of Cdc12 (G247E) can be readily explained if binding to CTP is sufficient to pass quality control.

Why does quality control fail to prevent incorporation of the mutant Cdc11 and SEPT12 proteins in Cs– yeast and infertile men, respectively? Haploid spermatagonia express only one of the two parental sepsin alleles but share a common cytoplasm (Dym and Fawcett 1971). Septin mRNAs and/or proteins diffuse throughout, as evidenced by the facts that the annuli in sept4 null sperm from a SEPT4/sept4 heterozygous male are indistinguishable from their SEPT4 counterparts (Ihara et al. 2005), and annuli were missing in all of the sperm from the SEPT12(D182N)/SEPT12 man (Kuo et al. 2012). Perhaps quality control acts at the level of sepsin incorporation into octamers, rather than octamer incorporation into filaments, such that when newly translated sepsins are assembling into new octamers, nucleotide-bound subunits are preferentially incorporated at the expense of nucleotide-free mutant proteins. If a sepsin-encoding transcript does not diffuse far from its nucleus of origin prior to translation, then subsequent octamer assembly may occur locally, and a mutant, nucleotide-free protein translated near a mutant nucleus could evade quality control by incorporating into octamers at a subcellular location distinct from the site of assembly by wild-type octamers. Diffusion-based mixing of preassembled octamers within the shared cytoplasm would then allow mutant-containing octamers to interfere with ring assembly. This model would also explain the dominance of the mutant Cdc11 molecules in the putatively heterokaryonic cdc11 Cs– sepsin yeast mutants.

Reciprocal genetic suppression identifies residues that dictate compatibility at the G interface

We propose that nucleotide-free Cdc10(D182N) is prone to adopting at high temperatures a conformation that, like Cdc10•GTP, is unable to associate with wild-type Cdc3•GTP, due in large part to steric and/or electrostatic interference by the charged sidechain of Cdc3 Asp210. Consistent with this idea, preventing GTP hydrolysis by Cdc10 via directed mutation renders cells Ts– (Sirajuddin et al. 2009). After we identified the cdc3(D210G) mutant, compelling biochemical support for this model was provided by analysis of purified human SEPT7, which is unable to form a SEPT7•GTP homodimer unless Asp103—homologous to Cdc3 Asp210, and which contacts Asp103 of the other protomer across the G interface of a SEPT7•GDP dimer (Zent et al. 2011)—is mutated to Ala (Zent and Wittinghofer 2013).

Loss of GTP binding by sepsins in evolution and disease

Intriguingly, a wild-type fission yeast sepsin, Spn7, has a drastically altered pocket lacking nearly all of the GTP-binding motifs, yet is capable of assembling efficiently into heterooctamers (Onishi et al. 2010). Spn7 occupies the same terminal position in sepsin octamers as does budding yeast Cdc11, but is expressed only during sporulation, when
septins form higher-order structures that are clearly distinct from the filamentous rings formed in mitosis (Onishi et al. 2010). Spn7 may have acquired the ability to adopt a G- and NC-dimerization-competent conformation without binding GTP, as we effectively reproduced in the lab with mutants of Cdc3 and Cdc10. Doing so would presumably sacrifice any ability to explore in a GTP-hydrolysis-coupled manner a wider range of stable conformations, but higher-order septin assembly in sporulating yeast may not require such regulated dynamics. Alternatively, Spn7 may have evolved to bind a non-GTP nucleotide; indeed, nucleotide pools undergo dramatic developmental remodeling during sporulation, at least in budding yeast (Jakubowski 1986; Jakubowski and Goldman 1988).

Considering that one of the infertility-causing septin mutations causes the same substitution as in Cdc10 (D182N), our findings clearly suggest that altering the G dimerization partner of SEPT12(D197N) by mutation or overexpression might rescue human septin function, and provide a general perspective for the development of therapeutic interventions targeted to disorders arising from septin mutations. Alternatively, it may be possible to slightly alter the temperature of an affected tissue/organ (i.e., the testis) to bias the conformation of a mutant protein toward the native, functional form. From the opposite perspective, there may be no "permissive" temperature for higher-order septin assembly if more than one septin subunit is mutated, imposing multiple conformational constraints at distinct septin–septin interfaces within or between heterooligomers. Indeed, combining multiple Ts− septin alleles in the same haploid yeast strain typically results in synthetic lethality (Longtine et al. 1996).

GTP binding and temperature sensitivity in cytoskeletal heteropolymers

It is particularly informative to compare our septin results to analysis of mutants of yeast α- and β-tubulin, the subunits of the heterodimer building block of microtubules. The substitutions in heat-sensitive tubulin mutants mapped almost exclusively to the α–β heterodimer interface, whereas cold-sensitive mutants harbored substitutions in residues that make contacts with other heterodimers in the context of a polymer (Richards et al. 2000). We propose that de novo assembly of the building blocks of septin or tubulin polymers requires the one-time acquisition of a single conformation, whereas the dynamic processes of polymerization and depolymerization require the polymerization interfaces to cycle through a set of discrete conformations. As with α-tubulin, which binds GTP but never hydrolyzes or exchanges it, nucleotide binding by septins plays a structural role in building block assembly, but for septins this role is only necessary in certain situations. High temperature makes it difficult for septins with no (or the wrong) nucleotide to adopt the conformation required to assemble the building blocks, and cold temperature constrains conformational flexibility, making it harder for the building blocks to come together into higher-order structures.

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Higher-Order Septin Assembly Is Driven by GTP-Promoted Conformational Changes: Evidence From Unbiased Mutational Analysis in *Saccharomyces cerevisiae*

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Figure S1  Heat- and cold-sensitive phenotypes and Cdc12 protein abundance in *cla10* mutants. (A) The *cla10* strains K3534, K3535, K3536, and K3538 were streaked to YPD and cultivated at the indicated temperature. (B) Total protein was extracted from the cells in (A) grown at 30˚C, resolved on a 10% SDS-PAGE gel, and transferred to a polyvinylidene membrane, prior to exposure to rabbit anti-Cdc12 (Versele and Thorner 2004) and anti-Zwf1 (Sigma-Aldrich A9521) antibodies, consecutively. Fluorescent anti-rabbit secondary antibodies were used for detection. The background-corrected relative ratios of Cdc12 (47 kDa) to the loading control Zwf1 (58 kDa) were normalized to that of the *cla10-1* strain and are indicated below each lane.
Figure S2  Cold-sensitive *cdc11* mutants carry two copies of the genome.

(A) Left, a cold-sensitive representation of the *CDC11* ORF indicating positions (in nucleotides) of mutations (red) found in strains N84.06D, and the amino acids encoded by affected codons. Sequences of the wild-type allele are given above, mutant sequences below. Below position 1053, a Scal recognition site is present in the mutant allele. Right, a PCR product encompassing the 3’-most ~620 nucleotides of the ORF plus ~30 nt of 3’ sequence was incubated for 6 hr in buffer alone (“mock”) or digested with Scal, and resolved adjacent to a DNA ladder (GeneRuler DNA Ladder Mix, Thermo Scientific #SM0331) on a 1% sodium borate agarose gel containing ethidium bromide.

(B) As in Figure 2A, sequencing results from PCR products of the indicated genes flanking or nearby *CDC11* in strain N84.06D. Arrows indicate positions with multiple signals.

(C) PFGE of chromosomes isolated from the indicated strains, run adjacent to a ladder of *S. cerevisiae* chromosomes (Bio-Rad #170-3605). Labels on the left identify each chromosome in the ladder.

(D) As in Figure 4C, Array-CGH of copy number variation in strains C17.01D and N84.06D compared to BY4741. The extra black horizontal line in C17.01D corresponds to the average signal for probes in the region of SAK1 amplification, shaded in purple.