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Off-Target Effects of the Septin Drug Forchlorfenuron on Nonplant Eukaryotes

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The septins are a family of GTP-binding proteins that form cytoskeletal filaments. Septins are highly conserved and evolutionarily ancient but are absent from land plants. The synthetic plant cytokinin forchlorfenuron (FCF) was shown previously to inhibit budding yeast cell division and induce ectopic septin structures (M. Iwase, S. Okada, T. Oguchi, and A. Toh-e, Genes Genet. Syst. 79:199–206, 2004, http://dx.doi.org/10.1266/ggs.79.199). Subsequent studies in a wide range of eukaryotes have concluded that FCF exclusively inhibits septin function, yet the mechanism of FCF action in nonplant cells remains poorly understood. Here, we report that the cellular effects of FCF are far more complex than previously described. The reported growth arrest of budding yeast cells treated with 1 mM FCF partly reflects sensitization caused by a *bud4* mutation present in the W303 strain background. In wild-type (*BUD4*$^+$) budding yeast, growth was inhibited at FCF concentrations that had no detectable effect on septin structure or function. Moreover, FCF severely inhibited the proliferation of fission yeast cells, in which septin function is nonessential. FCF induced fragmentation of budding yeast mitochondrial reticula and the loss of mitochondrial membrane potential. Mitochondria also fragmented in cultured mammalian cells treated with concentrations of FCF that previously were assumed to target septins only. Finally, FCF potently inhibited ciliation and motility and induced mitochondrial disorganization in *Tetrahymena thermophila* without apparent alterations in septin structure. None of these effects was consistent with the inhibition of septin function. Our findings point to nonseptin targets as major concerns when using FCF.

The septins are a family of highly conserved GTP-binding proteins found in a wide range of nonplant eukaryotes and some algae (1–3). In mammals, septin heterooligomers localize to the plasma membrane at sites of cell division and morphogenesis, along cytoskeletal filaments of actin or tubulin, and surrounding intracytosolic pathogens, among other locations (4–6). In budding yeast, where septin-encoding genes were first identified, the five members of the septin family, Cdc3, Cdc10, Cdc11, Cdc12, and Shs1, interact to form linear, nonpolar heterooctamers in the order Cdc11/Shs1-Cdc12-Cdc3-Cdc10-Cdc10-Cdc3-Cdc12-Cdc11/Shs1, where either Cdc11 or Shs1 can occupy the terminal subunit position, with distinct effects on higher-order assembly (7, 8). These octamers polymerize longitudinally to form filaments and can bundle laterally or intersect orthogonally to generate fibers, rings, and meshes (7–10). Yeast septins in proliferating cells assemble into cortical filamentous rings at the mother-bud neck, where they act as scaffolds and membrane diffusion barriers to control morphogenesis and cell division (6, 11). Single rings formed during bud emergence expand into collars at the neck, which split into dual rings during cytokinesis, one of each of which is inherited by mother and bud (11). Rings disassemble and reassemble prior to bud emergence in the subsequent cell cycle. The Bud4 protein appears to help couple septins to the sites of Cdc42-mediated cellular polarization (12–14). However, the molecular details of the regulation of higher-order septin assembly dynamics remain unclear.

Additional insight comes from alterations in septin localization accompanying genetic manipulations in yeast. The deletion of both *SHS1* and *CDC11* results in the assembly of the remaining septins into elaborate higher-order structures primarily associated with rings at the bud neck but also found at the cortex away from the neck (15), suggesting that there normally are constraints on higher-order septin assembly that act on the subunits at the filament polymerization interface. The mutation of various nonseptin yeast genes, including Cdc42 and its effectors, also frequently induces ectopic septin structures (16, 17), as does overexpression of nonseptin genes, such as Bud4 (12) or its orthologue from *Candida albicans* (Int1) (18). Some mutations even eliminate certain normal septin structures: deletion of *BUD4* causes the split septin rings that form during cytokinesis to disappear abnormally quickly, as does deletion of septin gene *CDC10*, at least in certain strain backgrounds (19). Importantly, most of these manipulations do not cause overt cytokinesis or growth defects. Thus, ectopic structures do not necessarily interfere with normal septin functions, and not all normal septin structures are required under all conditions.

In the study of cytoskeletal polymers, especially microtubules and actin microfilaments, the use of small molecules that directly alter polymerization kinetics *in vitro* and *in vivo* has been critical for the advancement of our understanding (20). These drugs, such as paclitaxel (originally named taxol) and nocodazole, are well characterized, and the mechanisms by which they affect their target molecules have been described (20). In fact, these drugs are so well understood that some of them (e.g., paclitaxel) are used clinically as effective cancer therapeutics (21). The lack of an equivalent tool for septin research has hindered progress in the understanding of many aspects of septin biology.

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Forchlorfenuron [FCF; N-(2-chloro-4pyridyl)-N-phenylurea, also called CPPU] is a synthetic plant cytokinin that is commonly used to increase fruit size (22). FCF binds and competitively inhibits the catabolic cytokinin dehydrogenase CKO (23), resulting in prolonged elevation of intracellular cytokinin levels and, ultimately, larger fruits (23). In 2004, Iwase et al. reported that treatment of the budding yeast *Saccharomyces cerevisiae* with FCF inhibits growth and causes the formation of ectopic septin structures (24). These effects were rapidly reversible and did not affect other cytoskeletal elements (24). The ectopic structures contained all of the mitotic septins, suggesting that the structures were composed of polymers of native septin heterooctamers (24). The effects of FCF on the filamentous fungus *Ashbya gossypii* were subsequently examined and included, at 125 μM, reversible inhibition of growth at the hyphal tip and ectopic polymerization of septins into long, stable fibers (25). Striking electron microscopy (EM) images of fixed FCF-treated *Ashbya* cells revealed paired filaments sharing all of the known properties of septin filaments formed *in vitro* from purified proteins (26). In cultured mammalian cells, FCF increased the length and width of apparently filamentous septin structures in a reversible manner (27). Septin function was perturbed by this apparent stabilization of higher-order assemblies, as multiple cellular effects phenocopied mutation or depletion of septins (27). Importantly, at FCF concentrations that mimicked septin loss of function, cellular viability was unaffected, and FCF killed cells only at much higher concentrations (27).

These *in vivo* findings provided strong evidence that septin polymerization into higher-order structures is potently induced and/or stabilized in cells treated with FCF. However, they do not distinguish whether FCF acts directly on septins or acts indirectly through another cellular factor to promote higher-order septin assembly. To address this issue, purified mammalian septin complexes were exposed to 5 μM FCF, which appeared to promote the lateral association of septin filaments (27). Thus, some evidence points to direct FCF-septin interactions that modify interactions between septin polymers. Moreover, the thermal stabilities of individually purified human septins are altered by the presence of FCF (28), consistent with direct binding. Finally, *in silico* modeling demonstrates that FCF fits well within the septin GTP-binding pocket (28). In general, the available evidence suggests that GTP binding and hydrolysis play structural roles in promoting higher-order septin assembly (11, 29). Thus, FCF may influence septin conformations relevant to polymerization dynamics. However, *in vivo* support for this model currently is lacking.

Despite the lack of a detailed mechanistic understanding of its action on septins, FCF has become a popular tool for the study of septin function. Septins have been found to be involved in the mammalian hypoxic response (30), calcium signaling (31), and glucose uptake (32), as well as algal cell division (3) and muscle contraction in blood flukes (33), partly on the basis of experiments using FCF as an inhibitor of septin function. Two major untested assumptions in these studies are that septins from different species respond the same way to FCF, and that off-target effects of the drug are negligible at working concentrations.

In this study, we set out to describe the cellular effects of FCF in a comprehensive and unbiased fashion. We find that the response to FCF is more complex than has been previously assumed, in budding and fission yeast as well as in cultured mammalian cells and a ciliated protist. These findings lead us to conclude that the off-target (nonseptin) effects of FCF are nonnegligible and must be carefully considered in future studies.

MATERIALS AND METHODS

Cells, plasmids, and media. FCF (KT-30 [Santa Cruz Biotechnology] or C2791 [Sigma]) was dissolved in 100% ethanol to a 250 mM stock. MitoTracker red CMXRos (M7512; Life Technologies) was dissolved in dimethylsulfoxide (DMSO) to a 1 mM stock. Phloxine B (AC1894700; Fisher Scientific) was dissolved in water to a 1 mg/ml stock and used at a 10 μg/ml working concentration. $DioC_6$ (3,3′-dihexyloxacarbocyanine iodide; 318426; Sigma-Aldrich) was dissolved in 100% ethanol to a 0.5 mg/ml stock and used at 250 ng/ml.

Budding yeast (*Saccharomyces cerevisiae*) cells typically were grown in rich medium (YPD; 1% yeast extract, 2% tryptone or peptone, 2% dextrose) or synthetic media containing 2% dextrose (unless indicated otherwise) and lacking one or more amino acids or nucleotides (drop-out mixes; United States Biologicals) as appropriate to maintain plasmid selection to mid-log phase, and then they were switched into FCF-containing YPD. Solid media contained 2% agar (BP1423; Fisher Scientific). The wild-type strain BY4741 (*MAT***a** *ho gal2 mal2 mel flo1 flo8-1 hap1 bio1 bio6 mip1 his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*) (34) expresses full-length Bud4. Its isogenic *bud4Δ*::*kanMX* derivative (MMY1032) was obtained from the haploid deletion collection (35) and was verified by amplifying via colony PCR the 5′ region of the gene replacement (*kanMX*) cassette using primers U1-DMA (5′GATGTCC ACGAGGTCTCT3′) and KanB (5′CTGCAGCGAGGAGCCGTAAT3′). Following treatment with alkaline phosphatase (FastAP; EF0651; Fermentas) and exonuclease I (EN0581; Fermentas), the PCR product was sequenced directly with KanB via standard dideoxy methods to verify the gene-specific barcode within this region (36). Strain JTY3992 is a *CDC10-mCherry*::*kanMX* isogenic derivative of BY4741 (15). The wild-type laboratory strain W303-1A [*MAT***a** *ho mal ssd1-d leu2-3,112 trp1-1 can1-100 ura3-1 ade2-1 his3-11,1 rad5*(*G535R*) *ybp1-1 bud4*] (37) contains a mutant allele of *BUD4* in which a single-nucleotide deletion causes a frameshift at codon 820, resulting in 18 missense amino acids followed by a premature stop codon at position 839, truncating the protein by 609 residues (http://www.yeastgenome.org/cgi-bin/FUNGI/alignment.pl?locus=YJR092W).

The low-copy-number *ARS/CEN* plasmids pZL02 (*URA3 CDC10-mCherry*) and pML43 (*LEU2 CDC11-YFP*) (38) encode full-length, wild-type budding yeast septins carrying C-terminal fusions to fluorescent proteins. pZL02 was constructed by Zhu Li of Jeremy Thorner's laboratory in the following way: *CDC10-mCherry* with ~800 bp of sequence 5′ from the start codon was amplified by PCR from genomic DNA of JTY3992 and cloned into the XbaI and ClaI sites of pRS316. The high-copy-number (2μ plasmid origin) plasmid pOli1-HcRed (39) expresses from the constitutive *ADH1* promoter the red-emitting fluorescent protein HcRed fused to the signal sequence of Oli1. A fusion to green fluorescent protein (GFP) of the mitochondrial targeting sequence (first 52 amino acids) of Cit1 was expressed under the *CIT1* promoter from a low-copy-number *ARS/CEN* plasmid marked with *URA3* (40). Bud4 with a C-terminal fusion to a hexahistidine tag, hemagglutinin epitope tag, and ZZ (IgG-binding) tag was overexpressed using a high-copy-number *URA3*-marked plasmid (pP_{GAL}-*BUD4*; obtained from the Yeast ORF Collection; YSC3870; Thermo Scientific/Open Biosystems) in which expression is controlled by the galactose-inducible *GAL1/GAL10* promoter. Yeast transformations were performed using the Zymo Research frozen EZ yeast transformation II kit (T2001).

Fission yeast (*Schizosaccharomyces pombe*) cells of strain JCF109 (h^+ *ade6-M216 his3-D1 leu1-32 ura4-D18*) (41) were cultured in YE5S medium (0.5% yeast extract, 3% glucose, supplemented to 250 mg/liter with adenine, histidine, leucine, uracil, and lysine hydrochloride) at 30°C. FCF or ethanol was added to the same medium.

Human retinal pigmented epithelial (RPE-1) cells were grown to 75% confluence on sterile polystyrene petri dishes in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS) in a 37°C incubator with 5% CO_2. For FCF treatment, cells were rinsed

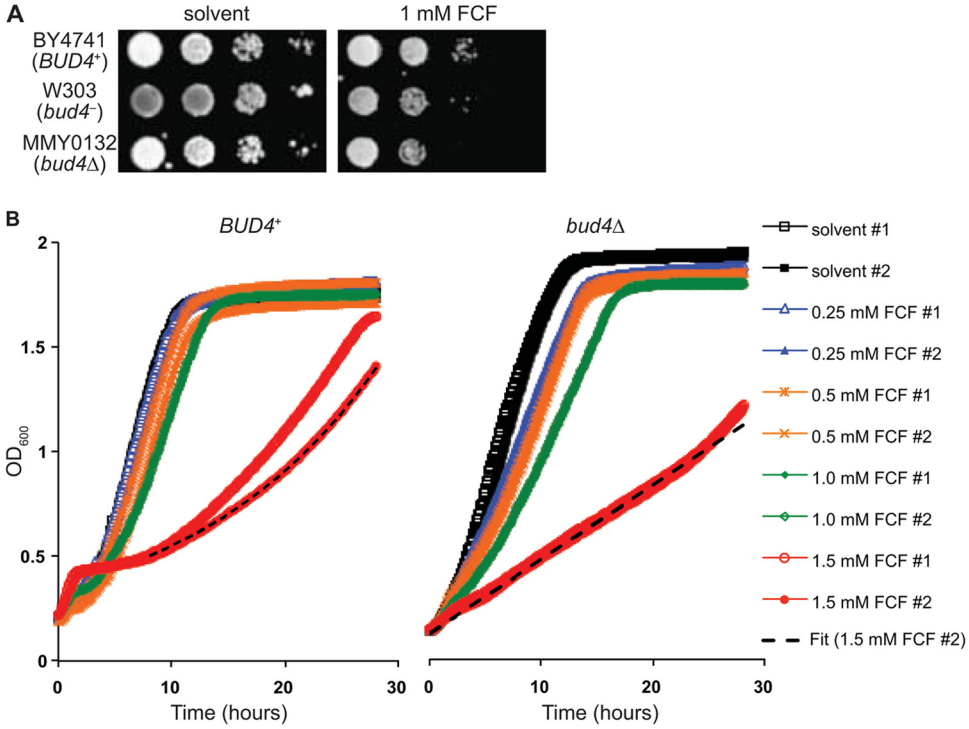

FIG 1 Loss of Bud4 function sensitizes *Saccharomyces cerevisiae* cells to growth inhibition by FCF. (A) Tenfold serial dilutions of the indicated strains were spotted on YPD plates containing solvent alone (0.4% ethanol) or 1 mM FCF and incubated at 22°C for 3 days or 5 days, respectively. (B) Duplicate (numbers 1 and 2) 200-μl liquid YPD cultures with 0.4% ethanol (solvent) or FCF at the indicated concentrations were inoculated at an optical density at 600 nm (OD_{600}) of 0.1 and monitored at 5-min intervals over the course of 28 h at 25°C. Dashed lines indicate fits of the indicated growth curves from the 7- to 28-h time points; see the text for details on curve fits.

and the medium was replaced with DMEM lacking FBS with either 0.02% ethanol or 50 μM FCF. After 2 h, mitochondria were stained for 30 min with 50 nM MitoTracker red CMXRos. Cells were rinsed 2 times with DMEM before visualization.

Tetrahymena thermophila strains expressing GFP-tagged septins (42) were maintained in PPYS (1% proteose peptone, 0.15% yeast extract, 0.01 mM $FeCl_3$, and 0.2 M NaCl) medium supplemented with 20 μM paclitaxel. The wild-type, untagged strain B2086 was used for MitoTracker staining. Tetrahymena cells were grown to mid-log phase (2×10^5 cells per ml) in SSP medium (2% proteose peptone, 0.1% yeast extract, 0.2% glucose, and 33 μM $FeCl_3$) without paclitaxel in a standing culture at 30°C. The expression of GFP-tagged septins was induced for 2 h after addition of 1 μg/ml $CaCl_2$. FCF or ethanol alone was added to the culture, and the cells were incubated for 1 h at 30°C. MitoTracker red CMXRos was added to 25 nM for 30 min before washing and visualization.

Measurement of culture density. Optical density measurements of yeast cultures were performed either in the cuvette reader of a NanoDrop 2000C or in flat-bottom Costar 96-well plates in a BioTek Cytation 3 imaging plate reader. Growth curve measurements were performed at 5-min intervals in the Cytation reader using continuous orbital shaking between reads and a temperature set point of 25°C. Fits to exponential growth equations were performed with GraphPad Prism software; goodness-of-fit values (R^2) ranged from 0.967 to 0.999.

Microscopy. Yeast, human, and MitoTracker-stained ciliate cells were visualized using an Olympus 60× Plan-Apo oil objective (numeric aperture [NA], 1.42) with an Advanced Microscopy Group EVOSfl microscope. LED filter cubes were GFP (AMEP4651), red fluorescent protein (RFP) (AMEP4652 for phloxine B, MitoTracker red, and HcRed), YFP (AMEP4654), or Texas red (AMEP4655 for mCherry). Budding yeast cells were spotted on 1% agarose pads or, for time-lapse imaging, loading into a CellASIC haploid yeast microfluidic plate (Y04C). Human cells were imaged directly on the petri dish. Fission yeast and MitoTracker-stained ciliate cells were spotted on glass slides and covered with coverslips (thickness no. 1) before imaging. Septin-GFP-expressing ciliate cells were fixed with 1% paraformaldehyde on cover glass (thickness no. 1.5H) before visualization by fluorescence microscopy with a Zeiss Observer D.1 microscope equipped with an AxioCam Mrm camera and a Zeiss Plan-Apochromat 63× objective lens (NA, 1.4).

RESULTS

Loss of Bud4 function sensitizes yeast cells to nonlethal inhibition of proliferation by FCF. We first set out to recapitulate the results of Iwase et al. (24) with regard to growth inhibition and septin localization in yeast cells exposed to FCF. Surprisingly, when grown at ~22°C, the wild-type laboratory strain BY4741 was distinctly less sensitive to growth inhibition by FCF (Fig. 1A) than the W303 strain used in the initial report (24). We considered the recent discovery that W303 harbors a loss-of-function allele of *BUD4* (19, 43) and tested a *bud4*Δ mutant of BY4741 (MMY0132). Indeed, loss of *BUD4* sensitized these cells to growth inhibition by 1 mM FCF (Fig. 1A). Notably, growth did not arrest completely in FCF: a subpopulation of cells of each genotype was able to form small colonies (Fig. 1A). In our hands, the effects of FCF in liquid culture were consistently more severe than those on solid media regardless of the genotype (data not shown). At high FCF concentrations, we frequently observed apparent precipitation of FCF on the surface of agar plates, which could result in a lower effective concentration. Therefore, we also assessed FCF effects on yeast growth in liquid culture. Measurements of growth in liquid culture taken at 5-min intervals over the course of 28 h at

25°C confirmed that in increasing concentrations of FCF (250 μM to 1.5 mM), growth was inhibited but did not cease (Fig. 1B). However, the subpopulation of cells capable of slow proliferation in high concentrations of FCF was clearly reduced in *bud4*Δ mutant cells (Fig. 1A and B). After 7 h, *BUD4*$^+$ cultures grew exponentially with a doubling time of 11.25 h (95% confidence interval, 11.20 to 11.31). In contrast, *bud4*Δ mutant cultures doubled every 14.06 h (95% confidence interval, 13.92 to 14.21). In the absence of FCF, *bud4*Δ mutant cultures grew similarly to the wild type. The MMY0132 doubling time was 3.26 h (95% confidence interval, 3.126 to 3.400), compared to 3.50 h for BY4741 (95% confidence interval, 3.385 to 3.626). The continued growth of FCF-treated cultures was consistent with our finding that the number of dead cells, as assessed by staining with the vital dye phloxine B, remained very low in *BUD4*$^+$ cultures inhibited by 18 h of exposure to 1 mM FCF (3.5% dead cells, $n = 589$ cells) compared to the solvent-only control (1.9% dead cells, $n = 611$ cells). Thus, wild-type cells clearly are susceptible to nonlethal growth inhibition by FCF, but the loss of Bud4 function confers a marked hypersensitivity.

FCF promotes the ectopic appearance of dynamic septin structures. We next examined the effects of FCF on septin localization in *BUD4*$^+$ and *bud4* cells using a plasmid-encoded mCherry-tagged version of the septin Cdc10 as a reporter. In cells of both genotypes, long ectopic septin fibers appeared at the cell cortex and in the cytoplasm (Fig. 2A). The *bud4* strains W303 and MMY0132 were biased toward cytoplasmic fibers, whereas the *BUD4* strain BY4741 exhibited primarily cortical localization of ectopic Cdc10-mCherry (Fig. 2A). Time-lapse microscopy of *bud4*Δ mutant cells exposed to 1 mM FCF showed that ectopic fibers often nucleated from the septin collar at the bud neck and either remained in contact with the cell cortex or detached to float in the cytoplasm (Fig. 2B). Figure 2B shows a fiber extending from the collar and detaching as the collar disappears (*bud4*Δ mutant cells make only very transient split rings at cytokinesis [19]). Fibers often abruptly vanished, usually concomitant with the assembly of a normal septin ring at the next site of budding (Fig. 2B). These observations suggest that FCF-induced fiber assembly involves some of the same factors that drive filament polymerization during normal ring assembly, and that fibers are dynamic structures capable of disassembly into subunits competent for immediate incorporation into normal structures.

Bud4 overexpression drives ectopic septin polymerization and interferes with septin function but does not sensitize budding yeast cells to FCF. FCF-induced ectopic structures were similar to ectopic septin structures formed upon Bud4 overexpression (Fig. 2C) (12), although an important difference is that when Bud4 was overexpressed, far more cells formed elongated buds (a hallmark of septin dysfunction) than when cells with normal levels of Bud4 were exposed to FCF (compare Fig. 2A with C). This result suggests that excess Bud4 interferes with the function of the endogenous septins to a greater extent than do the ectopic structures observed in these cells or in wild-type cells exposed to FCF. If FCF specifically interferes with yeast septin function, as opposed to the function of nonseptin targets, we predicted that treating *BUD4*-overexpressing cells with FCF would exacerbate the functional septin defects seen in these cells, leading to an increase in the number of cells with elongated buds. Instead, in the presence of FCF, *BUD4*-overexpressing cells were rounder (0 elongated of 50 total cells, 0%) than in its absence (17 elongated of 40 total cells, 42%; two-tailed $P < 0.0001$ by Fisher's exact test) (Fig. 2D). Considering that the elongated cellular morphology that accompanies septin dysfunction reflects an active response, i.e., a G_2/M cell cycle delay (reviewed in reference 44), to septin ring defects, these findings suggest that FCF inhibits yeast proliferation in ways that either interfere with the activity of this morphogenesis checkpoint or preclude it by arresting cells in pre-G_2 phases of the cell cycle. By comparison, *bud4* mutants in FCF grew more slowly and displayed more elongated cells (Fig. 2D), presumably because they are sensitized to FCF perturbation of septin function, triggering the G_2/M delay before nonseptin FCF targets have the opportunity to inhibit proliferation.

Low concentrations of FCF inhibit budding yeast proliferation without inducing ectopic septin structures. Studies in mammalian cells indicate that FCF stabilizes septin structures at much lower concentrations than those that inhibit cell proliferation and cause cell death (27). We analyzed BY4741 cells exposed to a series of increasing FCF concentrations to determine whether the same was true in *BUD4*$^+$ yeast. On the contrary, yeast cultures exposed to 0.5 mM FCF for 24 h displayed clear evidence of growth inhibition, whereas ectopic septin structures were detectable only in cells exposed to ≥1 mM FCF (Fig. 2A and E and data not shown). In the growth-inhibited cultures with only normal septin structures, cells were of normal, round morphology (Fig. 2D and data not shown). These results strongly suggest that cellular processes distinct from septin-dependent functions are affected at these lower FCF concentrations.

FCF inhibits fission yeast proliferation and does not phenocopy septin mutants. Unlike in budding yeast, septin proteins are not required for mitotic proliferation in fission yeast (*Schizosaccharomyces pombe*) cells (45–48). Although septin rings form at the site of cytokinesis in fission yeast cells, septin mutants lacking septin rings display only a slight delay in cell separation, manifested morphologically as a subset of chained cells in otherwise normal-looking cultures (45–48). If FCF specifically perturbs septin function in *S. pombe*, FCF should cause a slight inhibition of culture growth by wild-type cells, accompanied by the appearance of chained cells. In stark refutation of this prediction, the growth at 22°C of wild-type fission yeast cultures was noticeably inhibited by all FCF concentrations tested, with severe inhibition occurring at concentrations above 500 μM (Fig. 3A). In 100 μM FCF, where growth inhibition was slight, cells were slightly shorter and rounder, and this trend continued through the higher FCF concentrations tested (Fig. 3B), nothing like the reported phenotypes of septin mutant cells cultured under the same conditions without FCF (48). We conclude that, as on wild-type budding yeast cells, FCF exerts profound negative effects on the ability of wild-type fission yeast cells to proliferate that cannot be explained by loss of septin function.

FCF induces mitochondrial fragmentation and loss of mitochondrial membrane potential. We considered various nonseptin targets of FCF whose perturbation could rapidly inhibit proliferation and decided to investigate mitochondrial status in FCF-treated cells. Mitochondria are dynamic, essential organelles comprising elaborate reticular networks that are known to fragment into more punctate structures upon a number of cellular stresses (49). We transformed wild-type budding yeast cells with a plasmid encoding RFP fused to the mitochondrial targeting sequence of Oli1, the c subunit of the F0 ATP synthase (39). We then treated cells with 1 mM FCF or solvent alone and visualized the

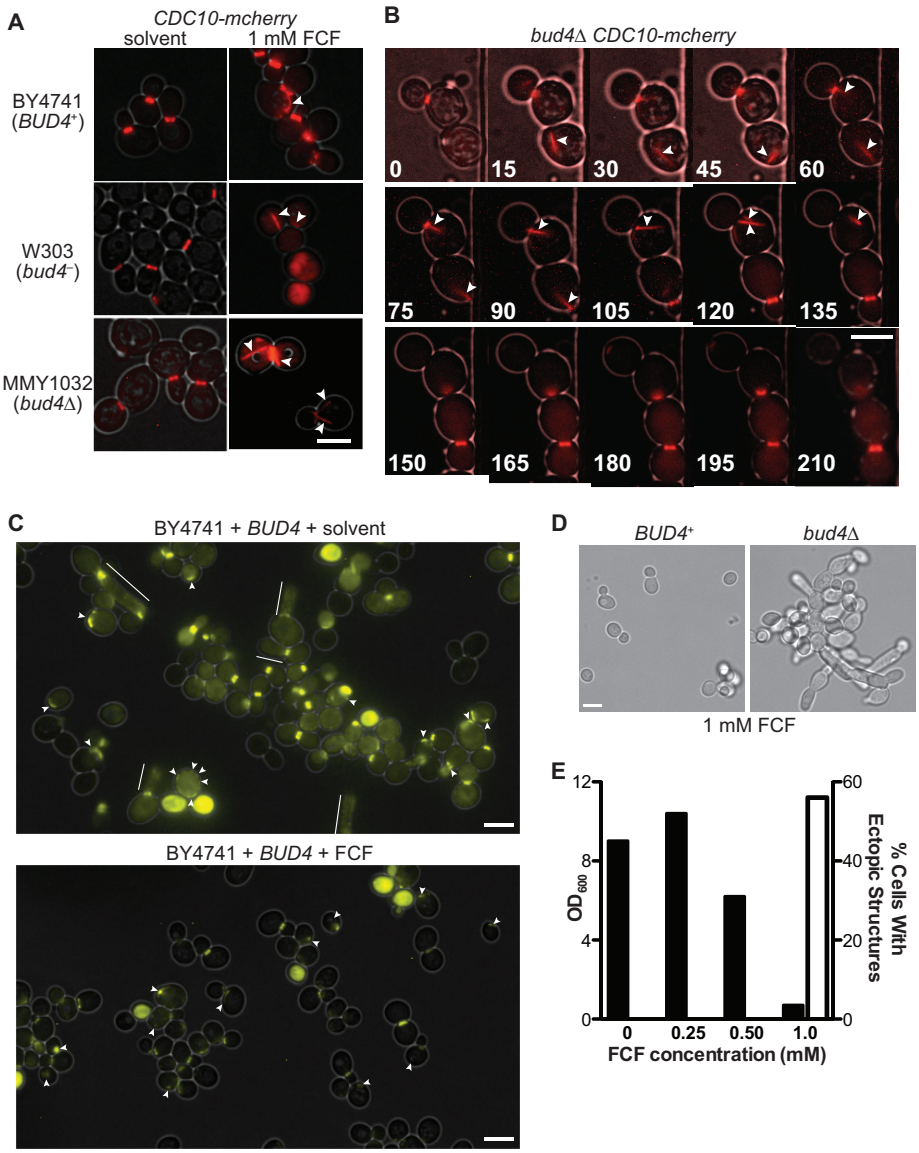

FIG 2 Ectopic septin structures in *Saccharomyces cerevisiae* are not responsible for growth inhibition by FCF. (A) Cells of the indicated strains carrying the Cdc10-mCherry-expressing plasmid pZL02 were grown to mid-log phase at 22°C in selective synthetic medium, loaded into microfluidic chambers, and exposed to a constant flow (85 μl/h) of the same medium containing 0.4% ethanol (solvent). After 1 h, the cells were imaged, and medium containing 1 mM FCF was introduced at the same flow rate. The cells were imaged again after 2 h. Shown are overlays of transmitted light images (to show cell outlines) and epifluorescence micrographs of mCherry fluorescence. Arrowheads indicate ectopic septin structures. (B) MMY0132 cells shown in panel A were imaged at 15-min intervals over a 3.5-h time course of exposure to 1 mM FCF. Inset numbers indicate time in min. Arrowheads indicate ectopic septin structures. (C) BY4741 cells expressing Cdc11-YFP from plasmid pML43 and overexpressing Bud4 from plasmid pP_{GAL}-*BUD4* were exposed to solvent alone (0.4% ethanol) or 1 mM FCF for 18 h. Arrowheads indicate ectopic septin structures. Lines indicate elongated buds, a hallmark of septin dysfunction. (D) Cells taken from 1.0 mM FCF cultures shown in panel B were visualized by microscopy to assess elongated cellular morphology as an indicator of dysfunction. (E) A YPD culture of JTY3992 was split into four cultures to which no FCF (but 0.4% ethanol; indicated as the zero baseline) or the indicated concentration of FCF was added. After overnight growth at 22°C, the densities of the cultures were measured (OD_{600}; filled columns), and samples were washed and imaged as described for panel A to score the fraction of cells containing ectopic septin structures (open columns). At least 260 cells were examined for each condition. Scale bars, 5 μm.

mitochondria in living cells using fluorescence microscopy. Overnight exposure to FCF resulted in an obvious change in mitochondrial morphology: reticular networks dispersed and formed bright foci within the cells (Fig. 4A). Numerous ectopic septin structures, marked with Cdc11-YFP, accumulated in the same cells (Fig. 4A). Upon removal of FCF by washing the cells with FCF-free growth medium, these effects began to reverse (Fig. 4A and data not shown). Similar effects also were seen using a fusion of GFP to the mitochondrial targeting sequence of the Cit1 protein (Fig. 4B) or the dye MitoTracker red (data not shown) as independent reporters of mitochondrial morphology.

FCF-induced changes in mitochondrial morphology were accompanied by functional defects in this organelle, as evidenced after 18 h of exposure to 1 mM FCF by a reduction in mitochondrial labeling with $DiOC_6$ (Fig. 4C), a membrane potential dye (50). This reduction in membrane potential is similar to what has

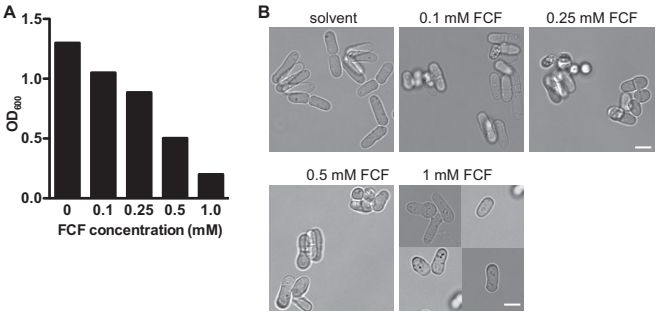

FIG 3 FCF inhibits the growth of *Schizosaccharomyces pombe* cultures and induces aberrant cellular morphologies that do not phenocopy septin mutation. (A) A YE5S culture of *S. pombe* strain JCF109 was split into five cultures to which various concentrations of FCF were added prior to overnight growth at 22°C, as described for Fig. 2E. (B) Samples from the cultures shown in panel A were imaged by transmitted light. Note that the 1 mM FCF image is a montage of images of smaller fields due to low cell density. Scale bars, 5 μm.

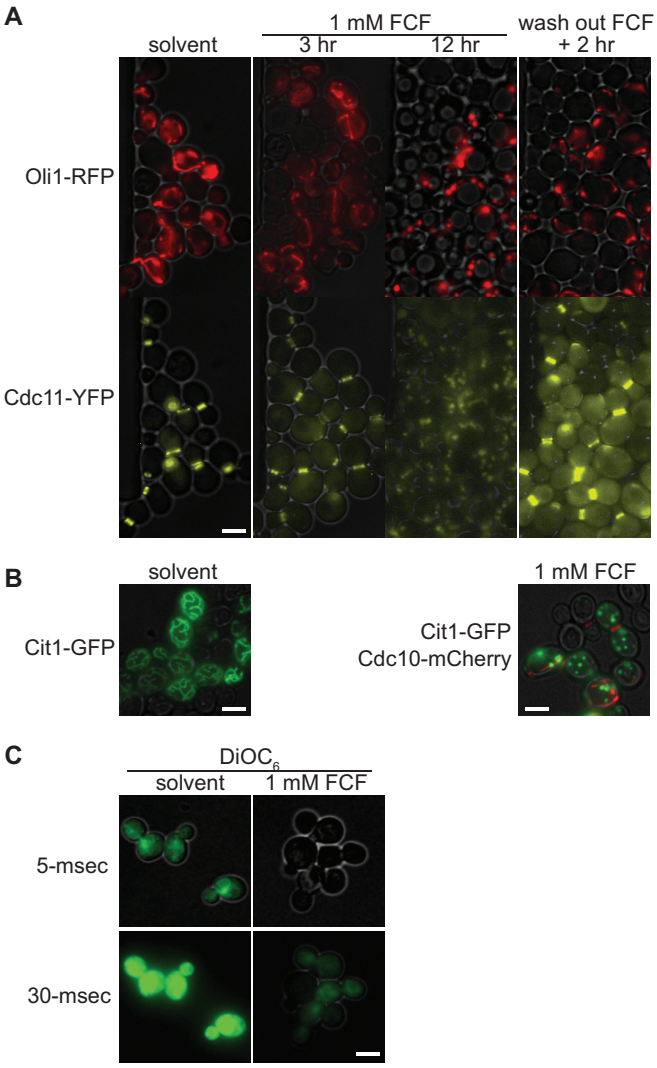

FIG 4 FCF induces mitochondrial fragmentation in *S. cerevisiae*. Shown are overlays of transmitted light images (to show cell outlines) and epifluorescence micrographs. (A) Cells of wild-type strain BY4741 carrying the Cdc11-YFP plasmid pML43 and the plasmid pOli1-RFP, which encodes the red-emitting protein HcRed fused to the mitochondrial targeting sequence of Oli1, were loaded into a microfluidic chamber and exposed to a constant flow (85 μl/h) of FCF-free medium before exposure to FCF-containing medium for the indicated times, as described for Fig. 2A. After 12 h, FCF-free medium was provided, and cells were imaged after an additional 2 h. (B, left) BY4741 cells expressing plasmid-encoded GFP fused to the mitochondrial targeting signal of Cit1 were grown overnight at 22°C in the presence of 0.4% ethanol (solvent). (Right) BY4741 cells expressing Cit1-GFP and coexpressing Cdc10-mCherry from plasmid pZL02 were grown overnight at 22°C in selective medium containing 1 mM FCF. (C) Wild-type cells of strain JTY3992 were grown for 18 h in the presence of 0.4% ethanol (solvent) or 1 mM FCF prior to staining with the mitochondrial membrane potential-sensitive dye $DiOC_6$ for 2 min. Images were captured with two different exposure times, as indicated, to demonstrate the difference in staining intensity. Scale bars, 5 μm.

been described for cytoplasmic petites that lack mitochondrial DNA (51). Considering that both cytoplasmic petites and most FCF-treated wild-type cells are viable but proliferate slowly, these results are consistent with FCF inhibition of mitochondrial functions that, under these culture conditions (i.e., 2% dextrose), are nonessential (e.g., oxidative phosphorylation).

FCF induces mitochondrial fragmentation in cultured mammalian cells. Taken together, our results suggested that in yeast, FCF causes mitochondrial fragmentation and inhibits growth at concentrations lower than those required for the induction of ectopic septin structures. In cultured mammalian cells, on the other hand, even prolonged (24-h) exposure to low FCF concentrations (≤62.5 μM) stabilizes septin structures and interferes with septin function without apparent effects on cellular proliferation or viability (27). The simplest explanation for the species-specific effects of FCF at low concentrations is that yeast mitochondria are particularly sensitive to perturbation by FCF, and nonseptin effects dominate the cellular response to the drug in these cells, whereas in mammalian cells septin effects dominate. If so, any effects of FCF on mitochondria in cultured mammalian cells should be manifested only at concentrations higher than those required for septin stabilization.

We used MitoTracker red CMXRos to label mitochondria in cultured human retinal pigmented epithelium (RPE) cells exposed to solvent alone or 50 μM FCF, a concentration reported to interfere with septin function in other cultured mammalian cells without inhibiting proliferation or causing cell death (27). Strikingly, this concentration of FCF had profound effects on the morphology of mitochondria, which appeared as bright puncta instead of the more diffuse network visible in cells exposed to solvent alone (Fig. 5). FCF-induced alterations in mitochondrial morphology in RPE cells reversed rapidly upon removal of FCF (data not shown). We conclude that the effects of FCF on mitochondrial morphology represent a conserved response to this small molecule by nonplant eukaryotes.

FCF effects in *Tetrahymena thermophila* do not phenocopy loss of septin function. Septins have been functionally linked to mitochondrial morphology in the protist *Tetrahymena thermophila*, where overexpression of the GFP-tagged septin Sep1 disrupted mitochondrial organization in some cells and led to the apparent autophagy of mitochondria (42). Sep1-GFP localizes to the cortical rows of mitochondria positioned adjacent to each motile cilium (42) (Fig. 6A). Knockouts of *Tetrahymena* septin genes display subtly mislocalized and disorganized mitochondria (42). Whereas mutants of the other two septins proliferate normally, ciliate cells lacking *SEP1* grow more slowly and show higher rates of mortality, but rates of motility are unaffected (42).

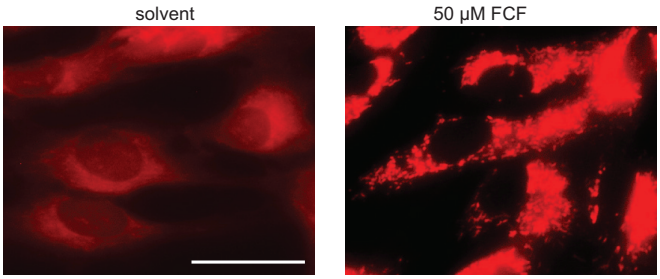

FIG 5 FCF induces mitochondrial fragmentation in cultured human cells. Human RPE-1 cells were exposed to 0.02% ethanol (solvent) or 50 μM FCF for 2 h prior to staining with MitoTracker red to visualize mitochondria. Scale bar, 100 μm.

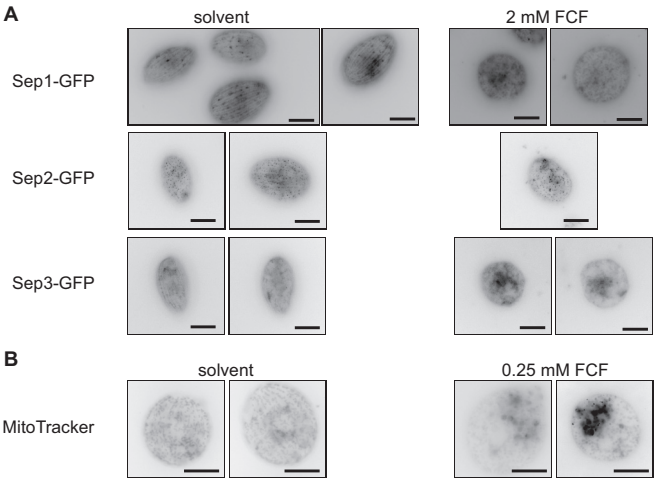

FIG 6 FCF causes loss of mitochondrial organization without ectopic septin structures in *Tetrahymena thermophila*. *T. thermophila* cells were exposed to 0.4% ethanol (solvent) or the indicated concentrations of FCF. (A) Cells expressing Sep1-GFP or Sep2-GFP, as indicated, exposed to solvent or FCF for 1 h. (B) Cells with untagged septins exposed to FCF for 20 min prior to MitoTracker staining. Images were converted to grayscale and inverted to improve visibility. Scale bars, 20 μm.

To test whether FCF alters mitochondrial morphology and/or higher-order septin structure in this organism, we exposed *Tetrahymena* cells to 2 mM FCF or solvent alone for 1 h and visualized mitochondria indirectly using Sep1-GFP and septins generally using Sep2-GFP. FCF had drastic effects on *Tetrahymena* physiology, causing a rapid (within 10 min) cessation of motility accompanied by cell rounding and an apparent loss of cilia (data not shown). These are not phenotypes of septin mutants (42) but are classic symptoms of stress response. At this concentration of FCF, the only ectopic septin structures observed were puncta of Sep2-GFP throughout the cells, but these also were present in solvent-exposed cells (Fig. 6A); thus, they likely are an artifact of overexpression. In contrast to the mild disorganization observed in cells lacking *SEP1* (42), the normal ciliary rows of mitochondria labeled by Sep1-GFP became grossly disorganized in FCF-treated cells (Fig. 6A). We used MitoTracker as an independent indicator of mitochondrial organization in cells with no tagged septin and observed gross FCF-induced disorganization in cells treated with as little as 250 μM FCF for 20 min (Fig. 6B). Longer treatments resulted in the same cessation of motility, cell rounding, and loss of cilia seen with 2 mM FCF in septin-GFP cells (data not shown). Triple-knockout mutants lacking all three *Tetrahymena* septins are less perturbed than single mutants (42). Thus, the cellular effects of FCF on *Tetrahymena* cells are much more severe than even the complete loss of septin function and occur without obvious accumulation of ectopic septin structures, pointing to a nonseptin target(s) of FCF with an essential role in normal ciliate physiology.

DISCUSSION

The findings we describe here provide compelling reasons to avoid the use of FCF as a tool to study septin function *in vivo* in ciliates, budding yeast, or fission yeast and probably explain why no published study has used FCF in *S. cerevisiae* in the decade since the initial report. While we were able to recapitulate the reported effects on ectopic polymerization of the septins, unless a sensitized genetic background (e.g., *bud4* mutant) is used, the concentration of FCF required to bring about these changes in septin structure is significantly higher than those causing drastic changes in septin-independent aspects of yeast physiology. The fact that overexpression of *BUD4* in untreated cells leads to the appearance of ectopic septin structures that are strikingly reminiscent of those found in FCF-treated cells points to Bud4 as a regulator of higher-order septin assembly but does not immediately suggest whether this protein generally promotes or restricts septin polymerization. Similarly, despite widespread use of FCF in the study of septins, we have only a very poor understanding of how it modifies septin assembly. Whether it can be used to inform us about normal roles of cellular factors in this process remains unknown. This stands in contrast to the historic effectiveness of drugs targeting other cytoskeletal polymers in gleaning new insights into regulation of cytoskeletal assembly and function.

Off-target responses to FCF have been documented in other cell types, most notably *A. gossypii*, in which 125 μM FCF caused, in addition to ectopic septin structures, reversible inhibition of growth at the hyphal tip, a gnarled cellular morphology, and the accumulation of abnormally thick septa, all common symptoms of general stress responses that are not seen in septin mutants of this fungus and occur even in septin mutant cells treated with FCF (25). At higher FCF concentrations (500 μM to 2 mM), the actin and tubulin components of the cytoskeleton also were perturbed (25). In organisms for which septin loss-of-function mutants are available or there are clear cellular phenotypes associated with septin dysfunction (such as elongated bud morphologies in *S. cerevisiae* and chained cells in *S. pombe*), it is possible to determine which are the off-target effects of FCF. In these cell types, FCF may be a useful tool to analyze higher-order septin dynamics, but conclusions about septin functions in various cellular processes based on FCF-induced effects should be made with the utmost caution. In systems in which septin genes are not easily mutated, numerous studies have attempted to address this issue using RNA interference-mediated depletion of septins, and in most cases this mimics the FCF effect of interest (30–32, 52). However, it has been well established that experimental activation of the RNA interference system via the introduction of synthetic small RNA molecules is associated with cytotoxicity and various indicators of general cellular stress (53–57), not unlike what we have documented for FCF in multiple cell types. Accordingly, until the septin-independent cellular effects of FCF are better understood, *in vivo* experiments using this compound must be accompanied by independent approaches to the study of septin function.

We examined mitochondrial structure in budding yeast, mammalian, and ciliate cells, and we found clear effects of FCF in each cell type. In each case, these effects are unlikely to be consequences of FCF-dependent alterations to septin function. In yeast, there is no evidence that septins localize to mitochondria or have any major role in maintaining mitochondrial structure or function. There are a few hints in the literature that septin dysfunction can affect other pathways that could be considered metabolic. For example, in *cdc10*Δ and *shs1*Δ cells, certain glucose-repressed genes are inappropriately derepressed (58) and a septin-dependent diffusion barrier at the midzone of zygotes regulates the timing of mixing of parental mitochondria (59), but these findings do not readily explain our observations. Notably, nutrient limitation, which could mimic mitochondrial dysfunction, drives colocalization of budding yeast septins with microtubules in long, fiber-like structures reminiscent of those induced by FCF (60). However, we determined, using a GFP-tagged tubulin construct, that FCF-induced septin fibers do not colocalize with microtubules (L. R. Heasley and M. A. McMurray, unpublished data).

In mammalian cells, the atypical septin ARTS localizes to mitochondria and is required for the induction of apoptosis in certain cell types and situations (61), but under our experimental conditions, the RPE cells we examined underwent essentially no apoptosis in the presence or absence of FCF (Heasley and McMurray, unpublished). Furthermore, the changes in mitochondrial morphology we observed upon FCF treatment were readily reversible upon removal of FCF, inconsistent with ARTS-initiated apoptosis. Thus, we find it unlikely that the alteration of ARTS function mediated the observed effects on mitochondrial morphology. Similarly, another splice variant of the *sept4* gene, M-septin, localizes to mitochondria but is expressed primarily in neurons (62), not epithelial cells. Finally, we analyzed a cell type in which septin-dependent alterations in mitochondrial organization have been reported, the ciliated protist *Tetrahymena thermophila* (42), and found that FCF caused changes in cellular function and septin localization that were distinctly different, and overall much more severe, than the reported effects of septin dysfunction.

The simplest interpretation of our data is that FCF targets a conserved, nonseptin molecule or set of molecules with important roles in overall cellular physiology. It is worth noting that FCF targets at least three distinct proteins in plants (23, 63), and it may be naive to imagine that for none of these would there be a nonplant protein sharing high structural similarity at the site of FCF binding. In principle, the same kind of biochemical approach used to identify CSBP as the major FCF-binding protein in plants (63, 64) could be applied to other organisms to isolate FCF-binding proteins. Notably, we failed to isolate a single reproducibly FCF-resistant mutant among a collection of ~4,500 viable deletion mutants of budding yeast (Heasley and McMurray, unpublished). However, more sophisticated genetic approaches may yield useful insights into FCF targets *in vivo*.

Our results in no way rule out direct FCF binding to septins, and FCF may still prove useful as an *in vitro* tool for the study of septin structure and function. If FCF indeed competes with GTP for occupancy of the septin nucleotide-binding pocket (28), it likely does so during *de novo* septin synthesis and folding, because preassembled yeast septins display only negligible *in vivo* exchange of bound GTP (65). Accordingly, in order to explain the rapid appearance of ectopic septin structures upon exposure of yeast cells to FCF, only a small number of FCF-bound septin proteins suffice to nucleate ectopic polymerization by GTP-bound septins that were synthesized prior to the introduction of FCF. Our results demonstrate that these polymers are subject to cell cycle-regulated disassembly, likely via the same poorly defined mechanisms that control normal septin ring disassembly.

There is clearly a persistent need for other small-molecule modifiers of septin structural dynamics. Recent studies have identified FCF derivatives that retain potent cytokinin activity (66) and may prove to be more specific toward septins *in vivo*. Alternatively, septin-targeting molecules may be among the compounds produced naturally by plants to resist septin-facilitated infection by certain fungi (67) or by pathogenic bacteria to counteract septin-based host defense mechanisms in metazoans (68). Finally, a very recent chemical genomics screen identified a synthetic small molecule that inhibited the growth of diploid budding yeast cells in which one copy of the septin-encoding gene *CDC12* was deleted (69). This compound, 1-ethyl-3-(4-methoxyphenyl)-6-methylpyrimido[5,4-e][1,2,4]triazine-5,7-dione, which in many ways resembles guanosine, was found to be ~5-fold more potent than FCF in inhibiting human septin function in a wound-healing assay using cultured cells (69). Despite this improved efficacy, however, the overall cellular response signature generated by this newly identified septin drug, i.e., the targeted biological process that emerges when considering all of the other yeast mutants that also were hypersensitive to the chemical, is one of iron homeostasis (69), in which septins have not been previously implicated. Thus, the search continues for a small-molecule septin inhibitor lacking significant off-target effects.

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