The fission yeast SEL1-domain protein Cfh3p: a novel regulator of the glucan synthase Bgs1p whose function is more relevant under stress conditions.

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Runing head: Cfh3p regulates Bgs1p

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In *Schizosaccharomyces pombe*, Bgs1/Cps1p is a β(1,3)glucan synthase required for linear β(1,3)glucan synthesis and primary septum formation. Here, we have studied the regulation of Bgs1p by Cfh3/Chr4p, a member of a family of conserved adaptor proteins, which resembles the chitin synthase regulator Chs4p from *Saccharomyces cerevisiae* and *Candida albicans*. *cfh3Δ* cells showed a genetic interaction with *cps1-191*, and Cfh3p co-immunoprecipitated with Bgs1/Cps1p. In the absence of *cfh3*$^+$, cells were more sensitive to digestion by glucanases, and both Calcofluor staining and glucan synthesis were reduced. We found that in a wild-type strain β(1,3)glucan synthesis was reduced under stress conditions. In the *cfh3Δ*, *cps1-191*, and *cfh3Δ cps1-191*strains, β(1,3)glucan synthesis was further reduced and growth was impaired under stress conditions, suggesting that Cfh3p and Bgs1p might play a role in ensuring growth in unfavourable environments. In a *cfh3Δ* mutant, Bgs1p was delocalized when the cells were distressed, but a blockade in endocytosis prevented this delocalization. Finally, we found that the SEL1 repeats are required for Cfh3p function. These results show that Cfh3p is a regulatory protein for Bgs1p and that its function is particularly necessary when the cells are undergoing stress.

INTRODUCTION

In *Schizosaccharomyces pombe*, the primary septum, composed of linear and branched β(1,3) glucan, is surrounded by a secondary septum with a composition similar to that of the lateral cell wall [branched β(1,3) glucan, β(1,6) glucan, α(1,3) glucan and mannoproteins (1,2)]. Bgs1/Cps1p is the β(1,3)glucan synthase responsible for the synthesis of linear β(1,3) glucan and the primary septum structure (3). In the absence of this activity, the cells are able to form remedial septa that do not contain linear β(1,3)glucan. These septa do not stain with low concentrations of Calcofluor and cannot be degraded by glucanases, so the cells remain chained, forming hyphal structures (3). In *S. pombe* there are four glucan synthase homologues, *bgs1*$^+$/*cps1*$^+$, *bgs2*$^+$, *bgs3*$^+$ and *bgs4*$^+$ (4-9). In the case of the *bgs1Δ* cells, apical growth takes place in two opposite directions, producing branched cells with a dichotomic growth. This phenotype is not observed in *bgs2Δ*, *bgs3Δ* or *bgs4Δ* mutants, thus suggesting that Bgs1p might play a specific role in the control of cell growth (3). Finally, Bgs1p is a component of the cytokinesis checkpoint, which coordinates mitosis with actomyosin ring contraction and septum synthesis (10,11).

The study of Bgs1p regulation should help us to understand the control of cell wall synthesis and cytokinesis in *S. pombe*. Bgs1p requires an active Septation Initiation Network (SIN), an assembled contractile actomyosin ring, and the type-V myosin Myo52p in order to localize properly at the division site (9,12,13). Regarding the regulation of biochemical activity, the PKC homologues Pck1p and Pck2p activate β(1,3)glucan synthesis in an unknown way (14) while the Rho1p GTPase is a direct activator of the β(1,3)glucan synthase catalytic subunit (15).

The cell wall is a morphogenetic determinant, but also an essential cellular structure that protects the organism against cell lysis in hypoosmotic environments. In *S. pombe*, the MAP kinase Spm1/Pmk1p pathway (also known as the cell integrity pathway) regulates growth and morphogenesis in response to multiple stresses, including hyper- or hypoosmotic conditions, nutrient limitation and cell wall-damaging compounds (16,17). Proper growth and morphology under hyperosmotic conditions also requires Skb1p and Skb5p, which are regulators of the Shk1/Orb2/Pak1p kinase (18,19). However, little is known about

the role of the cell wall as a protective element against hyperosmotic conditions, the presence of high concentrations of chloride ions, or nutrient limitation.

Here we examine the role of the Cfh3/Chr4 protein in cell wall synthesis and response to stress. Cfh3p shares significant similarity with the Chs4 proteins from *Saccharomyces cerevisiae* and from *Candida albicans*, which are chitin synthase regulators (20,21). In *S. cerevisiae*, regulation of the chitin synthase Chs3p by Chs4p is complex and still not well understood. Chs4p is required for the correct localization of Cfh3p at the bud neck by mediating its anchorage to septins through the adaptor protein Bni4p, but it also acts as a biochemical activator and is required for the stability of Chs3p at the plasma membrane (20,22-25). In *S. pombe*, no chitin synthesis occurs during vegetative growth (26-29), glucan being the main cell wall component, there is no Bni4p homologue, and septins are involved in cell separation but not in septum synthesis (30,31). We wanted to investigate whether Cfh3p played any role in cell wall synthesis and/or morphogenesis in the fission yeast. We found that in the absence of *cfh3*$^+$ the cells showed reduced β-glucan synthesis and impaired growth under stress conditions. These phenotypes were aggravated in a double *cfh3Δ cps1-191* mutant. Our results suggest that Cfh3p is a regulator of the β-glucan synthase Bgs1p whose presence is more critical when the cells are undergoing environmental stress. Cfh3p belongs to a conserved family of scaffold proteins characterized by the presence of tandem repeats of SEL1 domains, which are involved in signal transduction during different cellular processes (32). Here we found that the SEL1 domains in Cfh3p were required for its function.

EXPERIMENTAL PROCEDURES

Strains and growth conditions- All techniques for *S. pombe* growth and manipulation have been described previously (33; http://www.biotwiki.org/bin/view/Pombe/NurseLabManual). The relevant genotypes of the strains used are listed in Table S1 (supplemental material). Geneticin (G418, GIBCO-BRL) was used at 120 μg/ml.

Molecular and genetic manipulations- The general techniques were according to (34). The *cfh3*$^+$ gene was cloned from the genome of a wild-type strain by the *Gap Repair* technique (35). A *cfh3::KANMX6* deletion cassette in which the complete *cfh3*$^+$ ORF had been substituted by the KanMX6 gene, which confers resistance to geneticin (36), was used to transform the strains of interest. Correct integration was always assessed by PCR. Site-directed mutagenesis was used to introduce a *Not*I restriction site immediately downstream from the initial ATG codon. Three copies of the HA epitope, or the GFP, or Tomato RFP (37) proteins were cloned as *Not*I/*Not*I DNA fragments into the full-length and the truncated forms of *cfh3*$^+$. Functionality of the HA-Cfh3 and GFP-Cfh3 proteins was assessed by analyzing their ability to support growth of a *cfh3Δ* mutant on YES plates supplemented with $MgCl_2$ (data not shown), and to produce a multiseptation phenotype similar to that produced by Cfh3p when overexpressed (38 and our unpublished results). Site-directed mutagenesis was also used to substitute the CIIS aminoacids, corresponding to the prenylation motif, by a leucine residue and to introduce *Pma*CI restriction sites upstream and downstream from the SEL1 domains, which were eliminated by digestion with this enzyme and plasmid religation. The SEL1(1-2) truncated Cfh3 protein was produced by eliminating aminoacids 279 to 346; the SEL1(3-5) truncation was produced by eliminating aminoacids 443 to 543, and the SEL1(1-5) truncation was produced by deleting aminoacids 279 to 543. The complete aminoacid sequence of the modified proteins is shown in the supplemental figure S1.The accuracy of the constructions was assessed by DNA sequencing. These constructs, in which the *cfh3*$^+$ gene was under the control of its own promoter, were integrated at the *leu1*$^+$ *locus*. Double mutants were obtained by tetrad analysis. Combination of mutated alleles with HA-, GFP- or RFP-tagged proteins was performed either by plasmid transformation or by "random spore" selection from genetic crosses (33).

Protein techniques- For coimmunoprecipitation, cell extracts were obtained in extraction buffer (50 mM HCl-Tris, pH 7.5; 200 mM NaCl; 5 mM EDTA; 0.5% IGEPAL CA-630) with protease inhibitors (1 mM PMSF; 1 μg/ml Aprotinin, Leupeptin and Pepstatin). 5 mg protein from each extract was brought up to 330 μl with extraction buffer. Then, 30 μl from each sample was boiled in sample buffer and used to perform a Western blot. 300 μl of immunoprecipitation buffer (50

mM HCl-Tris, pH 7.5; 200 mM NaCl; 5 mM EDTA; 2% Triton X-100) with protease inhibitors was added to the remaining 300 µl of the extracts. 2.5 µl Rabbit anti-GFP antibody (Invitrogen, anti-GFP, serum) was added to the samples, which were incubated for 2 hours at 4 ºC in a tube rotator. Then, 50 µl of Protein A-Sepharose CL-4B beads (Pharmacia Biotech; 0.1 g/ml in IP buffer) was added to the samples, and the mixture was incubated overnight at 4 ºC. The beads were washed 3 times using IP buffer and once with Phosphate Buffered Saline, after which the beads were boiled in a final volume of 50 µl with Laemmli sample buffer (50 mM HCl-Tris, pH 6.8, 1% SDS, 143 mM β-mercaptoethanol, 10% glycerol) and centrifuged. Each supernatant was used to load two 6.5% polyacrylamide gels to be developed either with monoclonal anti-HA (12C5A, Roche; 1:4000) or with monoclonal anti-GFP (JL8, BD Biosciences; 1:1000). Secondary goat anti-mouse (Biorad) or anti-rabbit (Amersham) used at 1:10000 dilution and ECL (Amersham) were used to develop the blots. For Western blot, cells were broken in 50 mM TrisHCl, pH 8; 150 mM NaCl; 0.1% Triton X-100 supplemented with protease inhibitors. 100 µg protein from each sample was loaded in a 6.5% SDS-polyacrylamide gel and decorated with monoclonal anti-HA, anti-GFP, or anti-α-Tubulin (clone B-5-1-2, SIGMA; 1:10000) antibodies. In the case of the Bgs1 protein, the extracts were incubated in 1.6 M urea at 4ºC for 3 hours before being boiled in sample buffer.

Cell wall analysis- Analysis of cell wall composition was performed as described (39). Briefly, exponentially growing cultures were supplemented with 0.5 µCi/ml of D-[U-^{14}C]glucose (281 mCi/mmol; Amersham CFB96) and incubated for 6 hours at 25ºC. Total glucose incorporation was monitored by measuring the radioactivity in trichloroacetic acid-insoluble material. 100 µl-aliquots of total walls were incubated with 100 units of Zymolyase 100T (Seikagaku Kogyo Co. Ltd) or Quantazyme [recombinant β(1,3)glucanase; Quantum Biotechnologies Inc.] for 36 hours at 28°C. The samples were centrifuged and the supernatants and washed pellets were counted separately. The precipitate from the incubation with Zymolyase 100T corresponded to the α-D-glucan, and the supernatant corresponded to β-glucan plus galactomannan. The precipitate from the incubation with Quantazyme corresponded to α-glucan plus galactomannan, and the supernatant corresponded to (1,3)β–glucan. The difference between the precipitates of Quantazyme and Zymolyase 100T corresponded to galactomannan. The method did not distinguish β(1,6)glucan, which is a minor component of the *S. pombe* cell wall (1).

Enzyme preparation and ß(1,3) -glucan synthase assay- Cell extracts and glucan synthase assays were essentially as described previously (9). Early logarithmic phase cells grown at 25°C in the presence or the absence of 1 M KCl were washed with buffer A (50 mM Tris-HCl pH 7.5, 1 mM EDTA and 1 mM ß-mercaptoethanol), suspended in 100 µl of buffer A supplemented with 1 µl of 10 mM GTP-γ-S and broken with glass beads in a FastPrep FP120 apparatus (Savant; BIO 101. Three 15-second pulses at a speed of 5.0). Broken material was diluted with buffer A, the cell debris was removed by low speed centrifugation (800 g for 5 minutes) and the supernatant was centrifuged at 48,000 g for 30 minutes at 4°C. The pellet was resuspended in buffer A containing 33% glycerol and GTP-γ-S. Standard glucan assay mixture contained 5 mM UDP-D-[U-^{14}C]glucose (CFB102, Amersgham; 4x10^4 cpm/200 moles) and enzyme (15 µg protein) in a total volume of 40 µl. Reaction mixtures were incubated at 30ºC for 30 minutes, stopped by adding 2.5 ml of 10% trichloroacetic acid and incubated at 4ºC for at least 30 minutes before being filtered. Radiaoactivity was measured using a scintillation counter. All reactions were carried out in duplicate and data for each strain represents the average value from five independent cultures. The enzymatic unit was defined as the amount of enzyme that catalyzes the incorporation of 1µmol of glucose per minute at 30 ºC.

Digestion with glucanases- Sensitivity to digestion with glucanases was performed as described (39). Briefly, cells were resuspended in 2 ml of 50 mM citrate/phosphate buffer, pH 5.6, supplemented with different amounts of glucanases (Zymolyase 100T, Seikagaku, or Novozyme 234, Novo Industries) and incubated at 28ºC in a tube rotator. The $O.D._{600}$ was measured every 30 minutes during the incubation time, and the data were analyzed.

Microscopy- The observation of GFP-tagged proteins was performed on cells collected by filtration. Images were captured with a Leica DM RXA microscope equipped with a Photometrics Sensys CCD camera, using the

Qfish 2.3 program. Images were processed with Adobe Photoshop.

RESULTS

cfh3Δ mutants show a genetic interaction with mutants defective in septum synthesis- Deletion of the *cfh3*/*chr4*$^{+}$ gene did not lead to any apparent defect in cell morphology or in growth (38 and our unpublished results). To gain further information about the proteins functionally related to Cfh3p, we constructed double mutants involving *cfh3Δ* and the following mutants defective in different steps of cytokinesis: *cdc4-8*, *myo2-E1*, *myo3Δ*, and *myo2-E1 myo3Δ* (carrying mutations in type II-myosin components); *cdc11-119*, and *cdc14-118* (carrying mutations in genes from the Septation Initiation Network, which controls septum synthesis); *spn1::ura4*$^{+}$, *spn2::ura4*$^{+}$, *spn3::ura4*$^{+}$, *and spn4::ura4*$^{+}$ (deleted for septins), *chs2Δ* (defective in a chitin synthase-like protein), and *cps1-191*. The WT strain and the mutants carrying single or double mutations were streaked onto YES plates and incubated at different temperatures (from 22ºC to 37ºC). Only in the case of the *cfh3Δ cdc14-118* and the *cfh3Δ cps1-191* strains, did the double mutants show an enhanced thermosensitivity with respect to the WT and the corresponding single mutants (figure 1 A and results not shown). 1.2 M sorbitol supported the growth of the double mutants at the restrictive temperature (figure 1 A), suggesting that those strains had a defect in the cell wall.

bgs1/cps1Δ cells show a dichotomic growth and a multiseptation phenotype when they are kept alive in the presence of the osmotic stabiliser sorbitol (3). Accordingly, we wanted to study the morphology of the *cfh3Δ cps1-191*mutants with respect to that of the *cps1-191* strain in the presence and the absence of sorbitol. In YES medium at 32ºC, *cps1-191* cells were roundish or pear-shaped (figure 1 B). In the presence of 1.2 M sorbitol, *cps1-191* cells were rod-shaped, although some exhibited more than one septum (arrowheads in figure 1B), as has been described for other *bgs1* mutants (3). In YES and in sorbitol-supplemented YES media, the *cfh3Δ cps1-191* mutant exhibited long, multiseptated, branched cells with a dichotomic growth pattern. These cells were frequently dead, showing that this strain had a stronger defect than the single mutants, and that osmotic protection was not sufficient to correct this defect (figure 1 B). The double mutant exhibited this phenotype even at 25ºC (not shown). Thus, *cfh3Δ* showed a genetic interaction with some mutants affected in genes required for septum synthesis. Additionally, we found that a multicopy plasmid carrying the *cfh3*$^{+}$ gene under the control of its own promoter was able to improve the reduced growth of the *cps1-191* mutant at 32ºC (figure 1 C). All these results strongly suggest a functional relationship between Cfh3p and Bgs1p.

cfh3Δ mutants show a defect in glucan synthesis- Since the *cfh3Δ* mutant showed a genetic interaction with *cps1-191*, a mutant defective in the Bgs1p glucan synthase, we decided to determine whether the absence of the *cfh3*$^{+}$ gene might lead to a defect in glucan synthesis. We analyzed the growth of the WT, *cfh3Δ*, *cps1-191,* or *cfh3Δ cps1-191* strains in the presence of the glucan synthase inhibitors Caspofungin and Enfumafungin (40,41). We found that the *cfh3Δ*, *cps1-191* and *cfh3Δ cps1-191*cells were more sensitive than the WT at 2 μg/ml of Caspofungin and that the double mutant was the most sensitive of all the strains since it did not grow at 1.5 μg/ml, a concentration at which the single mutants were only partially sensitive. The mutants were also hypersensitive to 7μg/ml of Enfumafungin (figure 2 A). We next measured the β(1,3)glucan synthase activity in membrane extracts from the WT and the mutant strains incubated at 30ºC. As shown in figure 2 B, the specific activity was reduced to 85% and 84% in the *cfh3Δ* and the *cps1-191* strains, respectively, and to 71% in the double mutant. Although the differences in the specific activity between the strains were small, the tendency was the same in the 5 experiments that were performed, in which the mutants always exhibited a lower activity than the WT strain.

We then wanted to investigate whether the *in vitro* defect in the activity correlated with an *in vivo* defect in the synthesis of β(1,3) glucan. To do so, the sensitivity to digestion by glucanases of the WT cells was compared with that of the *cfh3Δ*, *cps1-191,* or *cfh3Δ cps1-191*cells. We found that the *cfh3Δ* cells lysed faster than the WT cells when they were incubated in the presence of 50 μg/ml of Novozyme; that the *cps1-191* cells lysed faster than the *cfh3Δ* cells, and that the *cfh3Δ cps1-191*double mutant cells were the most sensitive to digestion by the enzyme cocktail (figure 2 C). Similar results

were obtained when 5 μg/ml of Zymolyase 100T was used (results not shown). These results indicated that the cell wall composition was altered in all the mutant strains, probably due to a defect in β-glucan.

To confirm this, we analyzed the incorporation of radioactive [^{14}C]glucose into the cell wall polysaccharides of different strains incubated at 25ºC for 6 hours. As shown in figure 2 D, the percentage of glucose incorporation into β-glucan was 22.7% ± 1.91, 20% ± 2.15, 19.0% ± 1.86, and 13.7% ± 1.27 for the WT, *cfh3Δ*, *cps1-191*, and *cfh3Δ cps1-191* strains, respectively. Thus, β-glucan was reduced by 10% in the *cfh3Δ* strain; by 16% in the *cps1-191* strain, and by 39% in the double mutant. In the case of the *cps1-191* and *cfh3Δ cps1-191*cells, this decrease was compensated by an increased synthesis of mannan and, more significantly, by an increased synthesis of α-glucan. Accordingly, these two strains have more cell wall material than the WT and the *cfh3Δ* strains.

It has been shown that in *S. pombe* Calcofluor binds linear β-glucan, which is present mainly in the primary septum, and that *bgs1/cps1Δ* cells do not synthesize this structure, so the cells do not stain with low concentrations of this dye (3). We stained cells from the WT and the *cfh3Δ* strains with 0.125 μg/ml of Calcofluor and we found that at this concentration the dye was able to stain the septa of the WT cells efficiently, while in the *cfh3Δ* cells the septa only exhibited a faint staining (figure 2 E). These results show that the Bgs1p activity is compromised in the *cfh3Δ* cells.

The growth of the cfh3Δ, cps1-191 and cfh3Δ cps1-191 strains is impaired under certain stress conditions- During this work, we realized that *cfh3Δ cps1-191* cells showed the strongest morphological phenotype when the cells were entering the stationary phase, or exiting it, and that most of the cells died if they were kept on plates at 25ºC for more than 4 days. In order to capture this observation, we incubated the same number of cells from the WT, *cfh3Δ*, *cps1-191* and *cfh3Δ cps1-191* strains in liquid YES medium at 25ºC. Samples were taken from each culture after 4 or 7 days of incubation and 3x10^4 cells and serial 1:4 dilutions were spotted onto YES plates and incubated at 25ºC for 3 days before being photographed. As shown in figure 3 A, the survival of the *cfh3Δ cps1-191* cells was very low after they had been in the stationary phase, and that of the single *cps1-191* cells was also reduced with respect to that of the WT strain. This result suggested that the *cps1-191* and the *cfh3Δ cps1-191* cells were sensitive to nutritional stress. We therefore analyzed whether the *cfh3*, *cps1-191* and *cfh3Δ cps1-191* strains were sensitive to other sources of stress. We included a *cwg1-1* mutant, which carries a point mutation in the *bgs4*$^+$ glucan synthase homologue (4) and a *spm1Δ* mutant, defective in the Spm1/Pmk1 MAP kinase pathway, which controls morphogenesis and stress (17). As shown in figure 3 B, at the permissive temperature (25ºC) the *cfh3Δ cps1-191* and the *spm1Δ* cells were hypersensitive to 7 mM Caffeine; the *cfh3Δ*, the *cps1-191* and the double mutant were hypersensitive to 0.2 M $MgCl_2$, and the *cfh3Δ*, the *cps1-191*, the double mutant, and the *spm1Δ* strains were sensitive to 1.4 M KCl. In all conditions, the *cfh3Δ cps1-191* cells were more sensitive to stress than the corresponding single mutants (figure 3 B). The *cwg1-1* cells behaved as the WT did (figure 3 B), suggesting that a defect in *bgs1*$^+$, but not in *bgs4*$^+$, renders the cells sensitive to stress.

We next analyzed the growth of the single *cfh3Δ* mutant and the *spm1Δ* strain on YES plates supplemented with different amounts of KCl, NaCl, $MgCl_2$, Caffeine, and H_2O_2 and incubated at 32ºC. We found that the *cfh3Δ* and the *spm1Δ* cells were more sensitive than the WT cells to the presence of 1.2 M KCl and 6 mM caffeine; the *cfh3Δ* and the *spm1Δ* cells were slightly resistant to 0.1M NaCl, and the *cfh3Δ* cells were hypersensitive to 0.1M $MgCl_2$ (see figure 3 C). We did not find differences in the growth of the WT and *cfh3Δ* strains when they were incubated in the presence of different concentrations of hydrogen peroxide (figure 3 C and results not shown), suggesting that caffeine and osmotic stress, but not oxidative stress, affects the growth of *cfh3Δ* cells.

Impaired growth of the cfh3Δ, cps1-191 and cfh3Δ cps1-191 strains under stress conditions is concomitant with a reduction in glucan synthesis- It is known that mutants in the Spm1/Pmk1 MAP kinase pathway show a strong defect in cytokinesis when incubated under stress conditions (17). We observed the morphology of WT, *cfh3Δ*, *cps1-191* and *cfh3Δ cps1-191* cells that had been growing on solid or in liquid YES medium or in these media supplemented with 1.2 M sorbitol or 1.0 M KCl incubated at 25ºC or 32ºC for different times.

For each strain, the morphology of the cells grown under stress conditions was similar to that observed when the cells were grown in YES medium. The morphology of a *cfh3Δ spm1Δ* double mutant was similar to that of the single *spm1Δ* mutant in all conditions (figure S2 and results not shown). These results showed that the sensitivity of the *cfh3Δ* and the *cps1-191* cells to stress was not due to a defect in cytokinesis.

Since the mutants under study showed a defect in cell wall synthesis, we wondered whether this defect was responsible for their sensitivity to stress. To address this issue, we analyzed the cell wall composition of the strains of interest in cells that had been incubated for 6 hours in the presence of 0.6M KCl. As seen in figure 4 A, we found that all the strains showed a reduced incorporation of [^{14}C]glucose into the cell wall as compared to the data obtained when the cells had been growing in YES medium (the dashed line in figure 4 A represents the value of incorporation for the WT strain grown in YES medium. Compare figures 4 A and 2 D). Regarding the β-glucan content, the percentage of glucose incorporation into this polymer when the cells were grown in YES with 0.6 M KCl was 19.8% ± 1.74, 17.2% ± 1.66, 14.7% ± 1.14, and 12.3% ± 1.65 for the WT, *cfh3Δ*, *cps1-191*, and *cfh3Δ cps1-191* strains, respectively. Thus, in the WT strain grown under stress conditions the level of β-glucan was reduced by 12% with respect to the amount of this polymer in the cells grown in YES medium; this reduction was 24% in the *cfh3Δ* strain, 35% in the *cps1-191*strain, and 45% in the double-mutant strain. The dotted line in figure 4 A represents the level of β-glucan in the WT cells grown in YES medium.

In order to rule out the possibility that this defect in glucan synthesis under stress conditions was due to a defect in the accessibility of the substrate to the enzyme, we measured the β-glucan synthase activity in membrane extracts from the WT and the mutant strains that had been incubated in the presence of 1 M KCl at 25ºC for 20 minutes. It can be assumed that in the *in vitro* analysis the substrate is fully accessible to the enzyme. As shown in figure 4 B, the glucan synthase activity of the extracts from the WT cells incubated in the presence of KCl was reduced by 50% with respect to that of the cells incubated in YES medium. Similarly, the glucan synthase activity from the mutant strains was reduced when the cells had been incubated under stress conditions, with respect to the activity shown by the same strains incubated in YES medium (figure 4 B). As happened when the cells had been incubated in YES medium, the activity values for the mutants strains were reproducibly lower than that obtained for the control strain (figures 4 B and 2 B).

In order to determine whether the effect on the cell wall synthesis was specific for KCl or whether it was a general response to growth in a stressing condition or in a medium of higher osmolarity than the standard one, we analyzed cell sensitivity to digestion with glucanases. We incubated cells from the WT, *cfh3Δ*, *cps1-191,* and *cfh3Δ cps1-191* strains for 6 hours in YES or YES supplemented with 0.6 M KCl, 1.2 M sorbitol or 0.125 M $MgCl_2$, and then we treated them with 50 μg/ml Novozyme or with 5 μg/ml Zymolyase-100T. We found that in all cases the cells lysed faster when they had been incubated under stress conditions than when they had been incubated in YES medium (figure 4 C and results not shown). These results show that cell wall synthesis in general, and β-glucan synthesis in particular, is significantly reduced when the cells are undergoing stress, and that the impaired growth of the *cfh3Δ*, *cps1-191,* and *cfh3Δ cps1-191* strains in the presence of stressing compounds is correlated with the low β-glucan content in their cell walls.

Cfh3p regulates Bgs1p localization at the plasma membrane- All the above results suggested that Cfh3p was a regulator of Bgs1p and that this regulation was more needed when the cells were undergoing some stress. In order to understand the nature of this regulation, we performed several experiments. First, we used Western blotting to determine the level of Bgs1p in WT and *cfh3Δ* cells that had been treated with YES medium alone or supplemented with 1M KCl for 15 minutes. The Golgi protein Cfr1p (42) was used as a loading control. As shown in figure 5 A, there was no difference in the amount or the mobility of Bgs1p in the WT or the mutants cells regardless of whether they had suffered osmotic shock or not. This result showed that Cfh3p was acting at a post-translational level. Next, we wanted to know whether Cfh3p formed a complex with the glucan synthase machinery. To elucidate this, we constructed a strain that carried GFP-Bgs1 and RFP-Cfh3 tagged proteins and observed it under the fluorescence microscope. We found that both proteins colocalized (not shown), suggesting that they could be in close contact. To confirm this,

we performed a co-immunoprecipitation experiment using strains that carried GFP-Bgs1, HA-Cfh3 or both tagged proteins. HA-Cfh3 was detected in the anti-GFP immunoprecipitates from the strain bearing both tagged proteins, but not from the control strains. Bgs1p and Cfh3p also co-immunoprecipitated when the cells had been incubated in the presence of 1M KCl for 15 minutes (figure 5 B). We also analyzed co-immunoprecipitation between Cfh3p and the Rho1p GTPase, which regulates glucan synthase activity (15). In this case, we used strains carrying GFP-tagged Cfh3p, HA-tagged Rho1p, or both proteins. As shown in figure 5 C, we detected co-immunoprecipitation between GFP-Cfh3p and HA-Rho1p when the cells had been incubated either in YES medium or in YES medium supplemented with 1 M KCl for 15 minutes (figure 5 C). As a control, we analyzed co-immunoprecipitation between GFP-Cfh3p and HA-Cdc42p (a membrane-associated Rho family GTPase) using the same conditions and failed to observe any association between these proteins (not shown). All these results strongly suggested that Cfh3p would interact with the Bgs1 glucan synthase complex. In order to see whether Cfh3p was regulating the activation of Bgs1p by Rho1p, we determined the level of active Rho1p using the Rhotekin binding assay (43). We found that the levels of active Rho1p were similar in both the WT and the mutant strain (results not shown).

Finally, we wanted to know whether Cfh3p was regulating Bgs1p localization. To check this, we analyzed GFP-Bgs1 localization in the WT or the *cfh3Δ* strain grown in YES or in 1 M KCl-supplemented YES for 15 minutes. The localization of Bgs1p was similar in both strains when they were incubated in YES medium (figure 5 D), although the signal in the *cfh3Δ* cells seemed to fade faster. When the cells were incubated in the presence of KCl, the GFP-Bgs1p signal localized at the poles and the septum in the WT strain, although in some cells the septal area seemed to be deformed and the signal seemed to spread along the new cell pole (see arrows in figure 5 D). By contrast, in about 80% of the *cfh3Δ* cells Bgs1p could not be detected either at the septum or at the poles (figure 5 D) after the osmotic shock. A similar result was obtained upon exposing the cells to 1.2 M sorbitol for 15 minutes (not shown). Then, we wanted to investigate whether the different behaviour of the GFP-Bgs1 protein in the WT and the mutant strains was a consequence of a different behaviour of actin in response to the osmotic insult. To address this question, we observed the distribution of coronin (44; a protein that associated with the actin patches. Crn1-GFP in figure 5 E) in the WT and the *cfh3Δ* cells that had been exposed to 1 M KCl for 15 minutes. We found that in both strains the distribution of the actin patches was similar, being mostly observed at the poles and the medial region of the cells. In both cases it was possible to detect some defect in the morphology of the septal area (arrow in figures 5 E) as was observed in the WT strain carrying the GFP-Bgs1 protein (figure 5 D). We then wished to know whether the absence of the GFP-Bgs1 signal in the *cfh3Δ* cells incubated with KCl was due to a defect in the delivery of the protein to the cell membrane or to its rapid endocytosis. As shown in figure 5 F, the GFP-Bgs1 fluorescent signal was observed at the poles and midzone of the *cfh3Δ* cells when they had been incubated in the presence of both 1 M KCl and the actin-depolymerising drug Latrunculin A for 15 minutes, suggesting that a blockade in endocytosis was able to compensate for the absence of Cfh3p. This result was confirmed by analyzing the localization of Bgs1p in *cfh3Δ end4Δ* cells exposed to KCl for 15 minutes (figure 5 F). In order to know whether Cfh3p regulated the stability of other proteins at the plasma membrane, we analyzed the localization of Bgs4p and Chs2p in the WT or the *cfh3Δ* strains that had been incubated in YES or YES supplemented with 1 M KCl for 15 minutes. We found that Bgs4p delocalized from the plasma membrane in the presence of KCl in both the WT and the *cfh3Δ* strains (figure S3), while Chs2p localized properly in both strains (figure S3), supporting the idea of Cfh3p being a specific regulator of Bgs1p.

The SEL1 repeats but not the prenylation signal are required for Cfh3p function- Cfh3p is a member of a family of adaptor proteins that is present through the biological scale and is characterized by the presence of several copies of SEL1 domains, which are a subfamily of the Tetratricho Peptide Repeat (TPR) domains (45). Additionally, Cfh3p has a C-terminal prenylation motif (CIIS. See figure 6 A for a scheme of the structure of Cfh3p). We eliminated several or all of the SEL1 domains, and the CIIS prenylation motif, in order to see whether they were relevant for the function of Cfh3p. We analyzed the stability of the truncated forms of Cfh3p by

performing Western blotting of the GFP-fused proteins. As shown in figure 6 B, all the Cfh3p variants were stable. Then, we observed under the fluorescence microscope cells that carried the full-length Cfh3 protein, Cfh3 proteins in which all or several SEL1 domains had been deleted, or the Cfh3 protein in which the prenylation sequence had been eliminated, all fused to the GFP. We found that all these proteins localized to the cell poles and medial zone (figure 6 C, and results not shown). Finally, we checked the relevance of these domains for Cfh3p function by analyzing the growth capacity of cells carrying the untagged mutated forms of Cfh3p on minimal medium plates supplemented with 0.2 M $MgCl_2$ incubated at 34ºC. As shown in figure 6 D, elimination of some or all of the SEL1 domains abrogated function while the protein lacking the CIIS motif was able to support growth in the presence of $MgCl_2$ (figure 6 D).

DISCUSSION

In this work we have characterized the function of the Cfh3 protein. Matsuo *et al*. (38) suggested that Cfh3p/Chr4p would be a regulator of Chs2p, a chitin synthase-like protein that lacks such activity and is required for the stability of the contractile actomyosin ring during its contraction (46,47), because they found that in a *chr4Δ* mutant Chs2p could be observed at the ring and in some cytoplasmic dots. The following results disagree with this conclusion. i) in our previous work (46,47) and in this work we observed that Chs2p localized at internal vesicles in both the WT and the *cfh3Δ* strain (figure S4), depending on the culture conditions; ii) we did not find any genetic interaction between the *cfh3Δ* and the *chs2Δ* mutants; iii) the *chs2Δ* mutant showed a genetic interaction with the myosin mutants (47) but the *cfh3Δ* mutant did not (this work); v) the localization of Chs2p under stress conditions was the same in the WT and the *cfh3Δ* mutant (figure S3); vi) the *chs2Δ* cells were not sensitive to stress conditions (figure S5).

Our results point to a role of Cfh3p as a protein required glucan synthesis, whose absence is more detrimental for growth under certain stress conditions. We found that in a *cfh3Δ* mutant there was a small reduction in the glucan synthase activity and in the amount of β-glucan. This reduction was similar to that found in the glucan synthase-mutant *cps1-191* (figures 2 B and 2 D). Although we cannot rule out some regulation of other glucan synthases, the facts that a mutant in the $bgs4^+$ glucan synthase was not sensitive to stress, that a low concentration of Calcofluor could not stain the septa in the *cfh3Δ* mutant, and that the morphology of the *cfh3Δ cps1-191* cells was similar to that of the *bgs1Δ* cells maintained alive with an osmotic support (3) suggest that the defect in the β-glucan synthesis in the *cfh3Δ* cells would be due to a defect in the activity of Bgs1p. A defect in this glucan synthase is expected to result in a small defect in the β-glucan content since Bgs1p is responsible for the synthesis of linear β-glucan (3), which is a minor component of the cell wall (1).

The *cfh3Δ*, *cps1-191* and *cfh3Δ cps1-191* strains had a reduced viability when the media were supplemented with some stress-inducing compounds. However, it seems that these cells did not die because of abnormal cytokinesis, as has been described for the *spm1Δ* mutants (17), but because of a reduced cell wall synthesis. A reduction in total cell wall synthesis has also been observed in different strains grown in the presence of sorbitol (3,4). Sorbitol acts as an osmotic stabiliser that protects cells with a defective cell wall against lysis, but it also produces stress in the cells and induces stress-associated responses (48,49). We found that a feeble Bgs1 activity produced a further defect with respect to that produced by stress in the WT strain. Thus, in the double *cfh3Δ cps1-191* mutant cells grown under stress conditions the amount of β-glucan was almost 50% of the amount of this polymer in the WT cells grown under standard conditions. This strong defect would be deleterious for the cells. These results highlight the relevance of proper cell wall synthesis in general, and a Bgs1p-mediated β(1,3)glucan synthesis in particular, for the survival of the cells under unfavourable conditions, which are often found in natural environments.

The growth patterns of the *cfh3Δ* and *spm1Δ* mutants were similar in some stress-inducing media, which suggested that Cfh3p could be part of the Spm1/Pmk1 MAP kinase pathway (16,17,50). However, the following results argue against this hypotheis: i) the *cfh3Δ* mutants did not show an altered cytokinesis when they grew under stress conditions (figure S2); ii) the *cfh3Δ* mutants did not show the *vic* phenotype (viability in the presence of the

immunosupresant and chloride), characteristic of mutants in this MAPK signalling pathway (51; figure S2); iii) in the absence of *cfh3*$^{+}$, Spm1p was efficiently phosphorylated when the cells suffered an osmotic shock (figure S2).

Bgs1p is a transmembrane protein (13). Under normal laboratory conditions, most of Bgs1p seemed to be localized at the plasma membrane in the absence of *cfh3*$^{+}$. Accordingly, the *cfh3Δ* cells were able to grow and only showed a small reduction in the synthesis of linear β-glucan. When cells are exposed to some stress condition, a change occurs in the physical state and molecular interactions in the membrane bilayer, influencing lipid packing and dynamics (52). Transmembrane proteins are thus expected to assume new conformations and associations and to change their interactions with membrane lipids (53). Under these circumstances, the Bgs1p glucan synthase was not observed at the cell periphery when Cfh3p was absent (figure 5 D). The effect of stress was abolished when endocytosis was prevented using latrunculin A or an *end4Δ* mutant (54). In animal cells, exposure to some stress-inducing agents accelerates endocytosis (55). Our results suggest that a similar response to stress can take place in *S. pombe*. Thus, it seems that Cfh3p could act as a scaffold for the stabilisation of Bgs1p at the plasma membrane so that the synthesis of linear β-glucan will be ensured even if the environment is not favourable. Therefore, Cfh3p carries out a hitherto undescribed regulation of the synthesis of this polymer.

The most relevant feature in the Cfh3p molecule is the presence of five SEL1 repeats. In *S. pombe*, there are only seven proteins that bear SEL1 domains. Four of these proteins (Cfh1p to Cfh4p; 38 and our unpublished results) share the highest similarity with the chitin synthase regulators ScChs4p and CaChs4p (20,21), which also bear SEL1 domains (see figure 6). Proteins bearing SEL1 domains share a modular structure with several α-helixes, which gives them a solenoid structure, although normally share low levels of similarity in their primary sequences (32). Thus, it is not surprising that Cfh3p is 26% and 28% identical to ScChs4p and CaChs4p, respectively, and that these identity values only increase to 29% and 30%, respectively, when the comparison is restricted to the SEL1 domains. Our results show that Cfh3p plays a role in the regulation of glucan synthesis, in agreement with the idea that in *S. pombe* Chs-like proteins have maintained a general function not related to chitin synthesis, as reported for Chs2p and Cfr1p (42,46,47). In this case, the function would be to ensure the stabilization of enzymes involved in cell wall synthesis at the plasma membrane. It will be interesting to determine whether each of the other Cfh proteins regulate the Bgs2p, Bgs3p and Bgs4p glucan synthases by stabilizing them at the plasma membrane or if any of the other Cfh proteins play some role in cell separation through an interaction with septins.

Cfh3p, ScChs4p, and CaChs4p also share the presence of a prenylation site. We found that the prenylation motif was not essential for Cfh3p function. In the case of *S. cerevisiae*, a Chs4 protein lacking the prenylation site was also found to be able to complement a *chs4Δ* mutant (22,25), although this domain seems to be required for a robust chitin synthase III activity (23). Deletion of the SEL1 domains in Cfh3p resulted in a loss of function. In *S. cerevisiae*, the region of Chs4p containing the SEL1 domains is able to complement a *chs4Δ* mutant (22,24,25). The region of the *Candida albicans* Chs4 protein that contains the SEL1 domains is able to complement the *S. cerevisiae chs4Δ* mutant (21). These results show that these domains are relevant for the function of this family of proteins. It is known that there are SEL1 domain-proteins acting as adaptors in multiprotein complexes involved in different cellular processes such as cell cycle control, ER-associated protein degradation or bacterial virulence (32). Our results, together with those found in *S. cerevisiae* and *C. albicans*, show that protein complexes involving SEL1-domain proteins participate in cell wall synthesis and morphogenesis in different organisms.

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FIGURE LEGENDS

FIGURE 1**. The *cfh3Δ* mutant shows a genetic interaction with mutants affected in septum synthesis.** (A) The cells from the indicated mutants were streaked onto YES plates and incubated at 32ºC for 2 days. (B) The cells from the indicated strains were cultured in YES or YES supplemented with 1.2 M sorbitol at 32ºC and photographed. (C) The cells from the *cps1-191* strain carrying either the pAU vector alone or the pAU vector bearing the $cfh3^+$ gene were streaked onto minimal medium without uracil and incubated at the indicated temperatures for three days before being photographed.

FIGURE 2**. The *cfh3Δ* mutants show a defect in glucan synthesis.** (A) $3x10^4$ cells and serial 1:4 dilutions from the indicated strains were spotted onto YES plates or YES plates supplemented with the indicated amounts of the β-glucan synthesis inhibitors Caspofungin and Enfumafungin and were incubated at 25ºC for four days before being photographed. (B) β(1,3)glucan synthase activity. Cells from the indicated strains were incubated in YES liquid medium at 25ºC and the glucan synthase activity was analyzed at 30ºC. Bars represent the specific activity average, calculated from five independent extracts with duplicates. The standard deviation is shown (┬). (C) Lysis of cell suspensions from the WT strain (diamonds) and the *cfh3Δ* (squares), the c*ps1-191* (triangles) and the *cfh3Δ cps1-191* (circles) mutants grown in YES medium at 25ºC. Cells were incubated at 28ºC in 50 mM citrate-phosphate buffer in the presence of the indicated concentration of Novozyme 234; samples were taken every 30 minutes and their $OD_{600\ nm}$ was estimated. The experiment was performed three times, with similar results. A representative experiment is shown. (D) Percentage of incorporation of radioactivity into the cell wall polysaccharides of the indicated strains grown in YES medium at 25ºC in the presence of [^{14}C]glucose. The grey-filled bars represent β-glucan, the black-

filled bars represent α-glucan, and the hatched bars represent the mannoproteins. For comparison, the dotted line marks the level of β-glucan in the WT strain. The experiment was performed 5 times with duplicates. The standard deviation for the total incorporation in the cell wall is shown (┬). (E) DIC and fluorescence micrographs of cells from the WT or the *cfh3Δ* mutants grown in YES at 32ºC and stained with 0.125 μg/ml Calcofluor.

FIGURE 3**. Growth of the *cfh3Δ*, *cps1-191* and *cfh3Δ cps1-191* strains is defective under stress conditions.** (A) $3x10^4$ cells and serial 1:4 dilutions from cultures from the indicated strains that had been incubated at 25ºC for four or for seven days were spotted onto YES plates and incubated at 25ºC for three days. (B) $3x10^4$ cells and serial 1:4 dilutions from the indicated strains were spotted onto YES plates or YES plates supplemented with the indicated concentration of caffeine, $MgCl_2$ and KCl and incubated at 25ºC for six days before being photographed. (C) Same as in (B) but the plates were incubated at 32ºC for 2 days.

FIGURE 4. **Cell wall synthesis is defective in the *cfh3Δ*, *cps1-191* and *cfh3Δ cps1-191* strains grown under stress conditions.** (A) Percentage of incorporation of radioactivity into the cell wall polysaccharides of the indicated strains incubated at 25ºC for 6 hours in YES medium supplemented with 0.6 M KCl and in the presence of [^{14}C]glucose. The grey-filled bars represent β-glucan, the black-filled bars represent α-glucan, and the hatched bars represent the mannoproteins. The dashed and the dotted lines mark the level of the radioactivity incorporated in the total cell wall and in the β-glucan in a WT strain grown in YES medium, respectively. The experiments were performed 5 times with duplicates. The standard deviation for the total incorporation in the cell wall is shown (┬). (B) β(1,3)glucan synthase activity. Cells from the indicated strains were incubated in YES (black-filled bar) or YES supplemented with 1 M KCl (grey-filled bars) for 20 minutes at 25ºC and the glucan synthase activity was analyzed at 30ºC. Bars represent the specific activity average, calculated from five independent extracts with duplicates. The standard deviation is shown for every value (┬). The black dashed line was drawn to highlight the value obtained for the WT strain grown in YES medium. (C) Lysis of cell suspensions from the WT strain grown in YES medium (circles) or YES medium supplemented with 1.2 M sorbitol (diamonds), 0.6 M KCl (triangles), or 0.125 M $MgCl_2$ (squares) at 25ºC for 6 hours. Cells were incubated at 28ºC in 50 mM citrate-phosphate buffer in the presence of 50 μg/ml of Novozyme 234, samples were taken every 30 minutes and their $OD_{600\ nm}$ was estimated. The experiment was performed three times with similar results. A representative experiment is shown.

FIGURE 5. **Cfh3p regulates Bgs1p localization.** (A) Upper panel: Anti-HA Western blot analyzis of the amount of HA-tagged Bgs1p in WT or *cfh3Δ* cells incubated with (+) or without (-) KCl for 15 minutes. Lower panel: anti-GFP Western blot to detect the GFP-fused Cfr1 protein used as a loading control. (B) Cfh3p and the glucan synthase Bgs1p co-immunoprecipitate. Cell extracts from strains carrying GFP-Bgs1 and/or HA-Cfh3 were analyzed by Western blotting using anti-GFP (α-GFP) or anti-HA (α-HA) monoclonal antibodies before (Extracts) or after immunoprecipitation (IP) with a polyclonal anti-GFP antibody. The cells had been incubated either in YES medium or YES supplemented with 1 M KCl for 15 minutes before being collected. (C) Cfh3p and the glucan synthase regulator Rho1p co-immunoprecipitate. Cell extracts from strains carrying GFP-Cfh3 and/or HA-Rho1 were analyzed by Western blotting using anti-GFP (α-GFP) or anti-HA (α-HA) monoclonal antibodies before (Extracts) or after immunoprecipitation (IP) with a polyclonal anti-GFP antibody. The cells had been incubated either in YES medium or YES supplemented with 1 M KCl for 15 minutes before being collected. (D) Bgs1p delocalizes in the *cfh3Δ* mutant after a stress shock. Fluorescence micrographs from WT or *cfh3Δ* cells carrying the GFP-Bgs1 fusion protein that had been incubated in YES medium or YES supplemented with 1 M KCl for 15 minutes before being collected by filtration. (E) Distribution of actin patches in cells treated with 1 M KCl for 15 minutes. Same as in (D) but the cells carried the Crn1-GFP protein and were incubated in the presence of 1 M KCl. (F) A blockade in endocytosis prevents Bgs1p delocalization from the membrane in the *cfh3Δ* cells under stress conditions. Same as in (D) but the *cfh3Δ* cells were either incubated in the presence of 1 M KCl and latrunculina A (left panel) or carried an *end4Δ* mutation (right panel) and were treated with 1 M KCl. The arrows point to cells exhibiting an abnormal septal area.

FIGURE 6. **The SEL1 domains are required for Cfh3p function.** (A) Schematic representation of the full-length Cfh3 protein, or different truncated versions of this protein, where the grey diamonds represent the SEL1 domains and the grey circle represents the prenylation motif. The *Saccharomyces cerevisiae* (Sc) and *Candida albicans* (Ca) Chs4 proteins have been included, for comparison. (B) The Cfh3p truncated proteins are stable. Anti-GFP (upper panel) and antitubulin (lower panel) Western blots of cell extracts from strains bearing the indicated versions of Cfh3p. (C) Localization of GFP-Cfh3p (left panel), GFP-Cfh3$_{\Delta SEL1-5}$ (central panel), and GFP-Cfh3$_{-CIIS}$ proteins. (D) 3x10^4 cells and serial 1:4 dilutions from the WT strain and the *cfh3Δ* mutant transformed with an empty integrative plasmid, or from the *cfh3Δ* mutant transformed with integrative plasmids bearing the indicated untagged versions of *cfh3*$^+$ were spotted onto minimal medium without leucine (left panel) or the same medium supplemented with 0.2 M $MgCl_2$ and incubated at 34ºC for 2 days before being photographed.

FIGURE 1

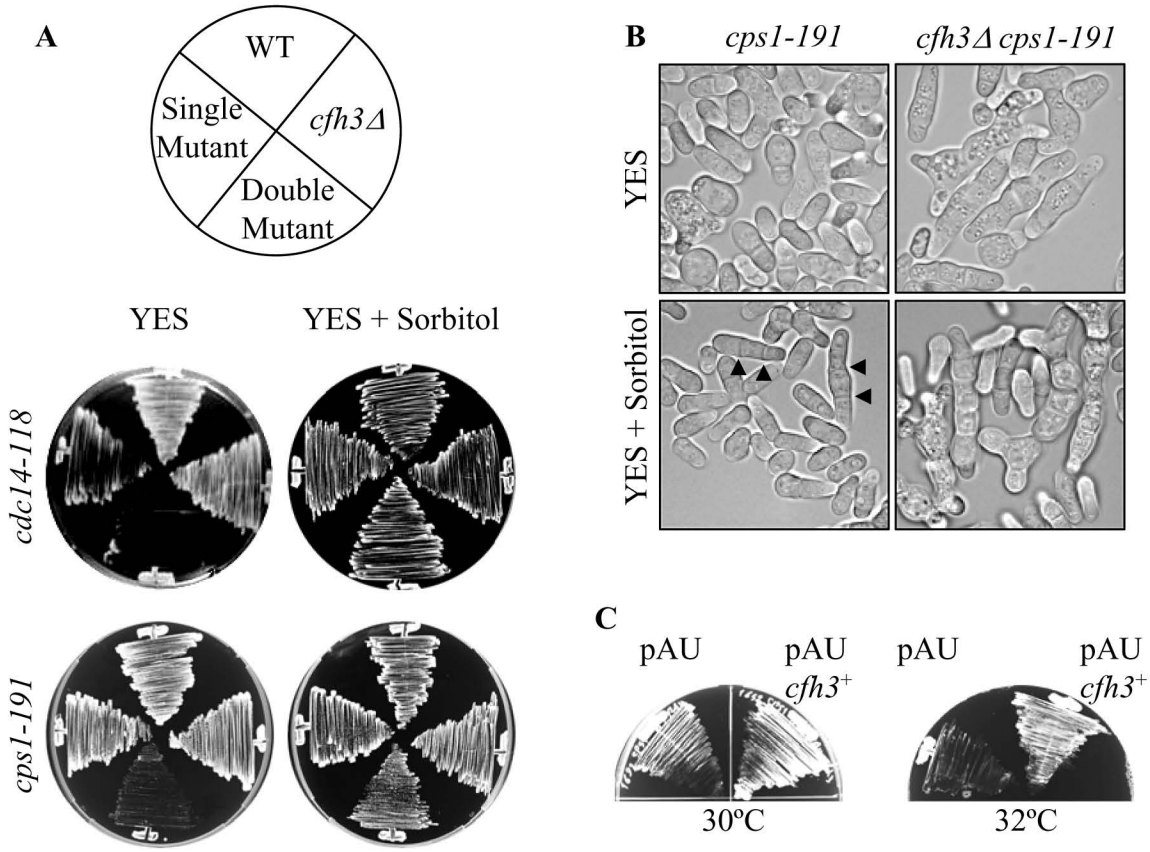

A
WT
Single Mutant
cfh3Δ
Double Mutant
YES
YES + Sorbitol
cdc14-118
cps1-191
B
cps1-191
cfh3Δ cps1-191
YES
YES + Sorbitol
C
pAU
pAU cfh3+
pAU
pAU cfh3+
30ºC
32ºC

FIGURE 2

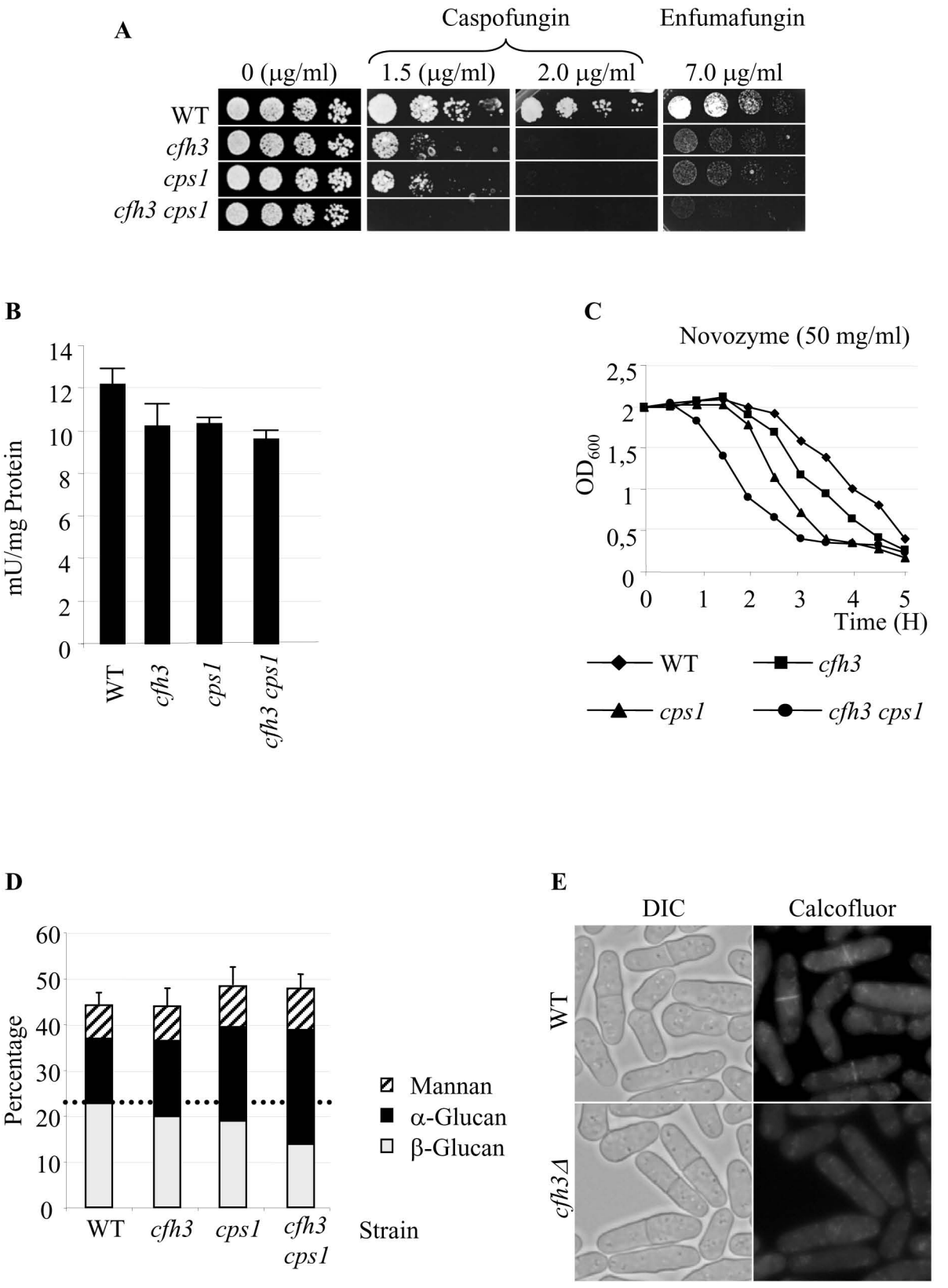

A
Caspofungin
Enfumafungin
0 (μg/ml)
1.5 (μg/ml)
2.0 μg/ml
7.0 μg/ml
WT
cfh3
cps1
cfh3 cps1
B
mU/mg Protein
14
12
10
8
6
4
2
0
WT
cfh3
cps1
cfh3 cps1
C
Novozyme (50 mg/ml)
OD600
2,5
2
1,5
1
0,5
0
0
1
2
3
4
5
Time (H)
WT
cfh3
cps1
cfh3 cps1
D
Percentage
60
50
40
30
20
10
0
WT
cfh3
cps1
cfh3 cps1
Strain
Mannan
α-Glucan
β-Glucan
E
DIC
Calcofluor
WT
cfh3Δ

FIGURE 3

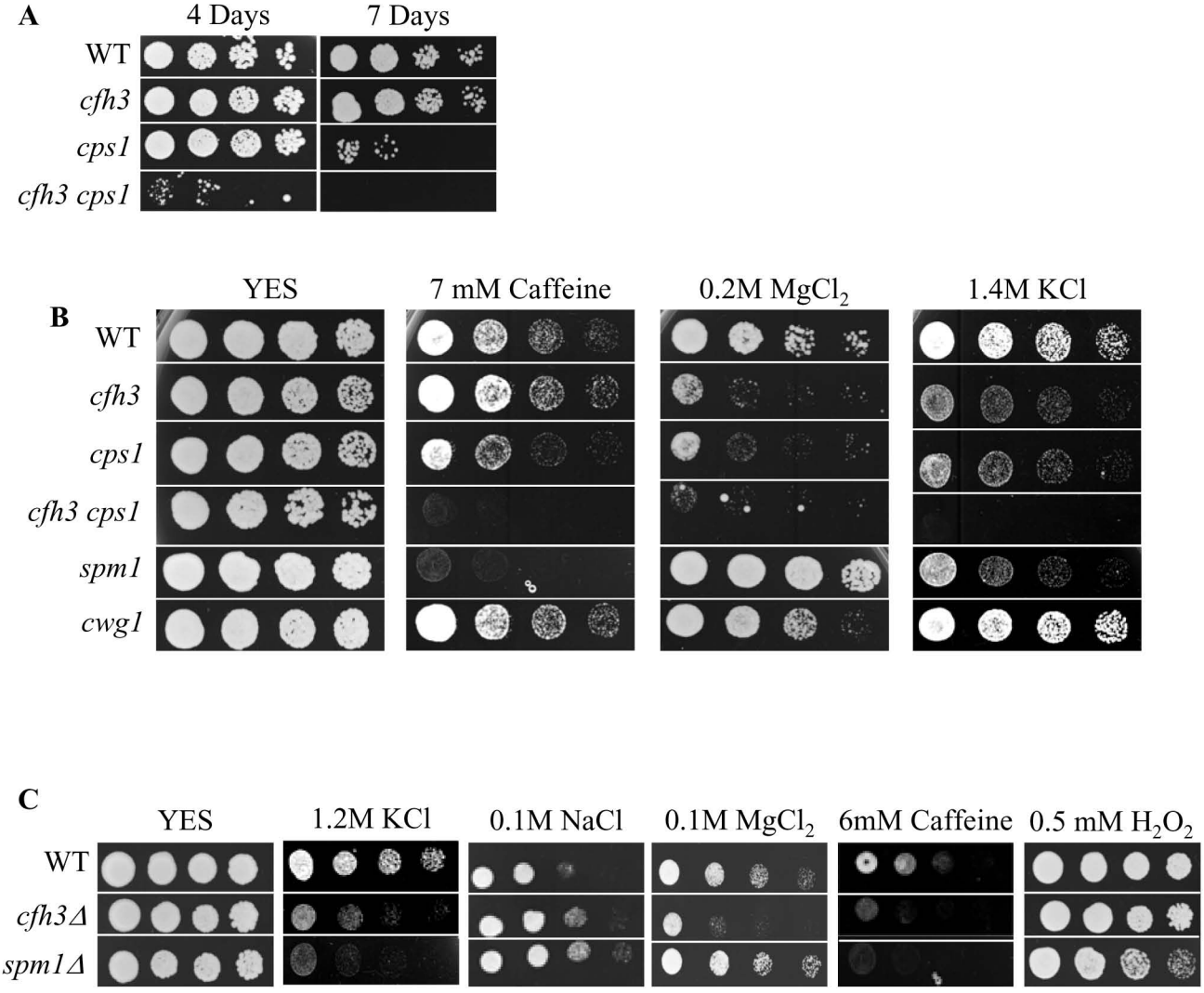
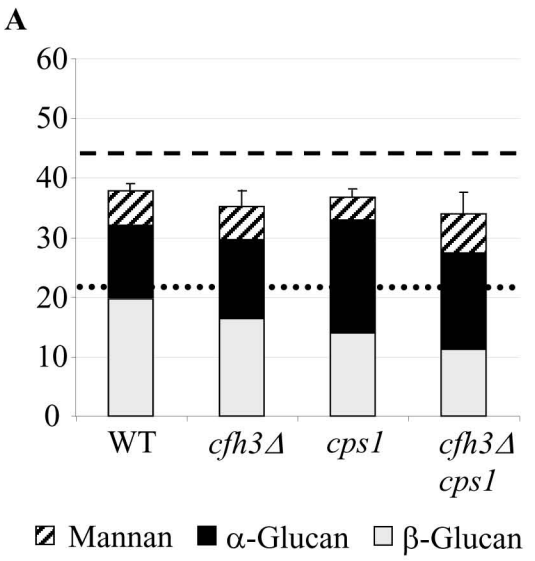
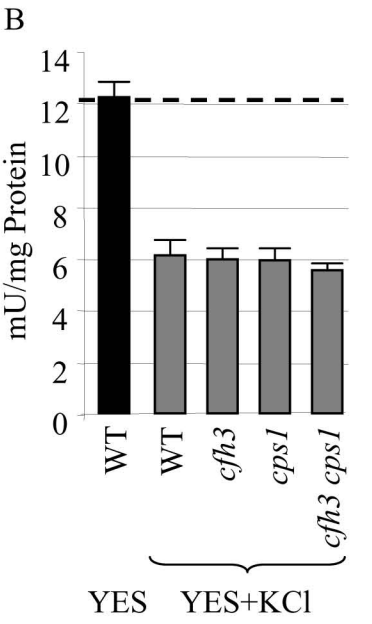

A
4 Days
7 Days
WT
cfh3
cps1
cfh3 cps1
B
YES
7 mM Caffeine
0.2M MgCl2
1.4M KCl
WT
cfh3
cps1
cfh3 cps1
spm1
cwg1
C
YES
1.2M KCl
0.1M NaCl
0.1M MgCl2
6mM Caffeine
0.5 mM H2O2
WT
cfh3Δ
spm1Δ

FIGURE 4


A
60
50
40
30
20
10
0
WT
cfh3Δ
cps1
cfh3Δ cps1
Mannan
α-Glucan
β-Glucan


B
14
12
10
8
6
4
2
0
mU/mg Protein
WT
WT
cfh3
cps1
cfh3 cps1
YES
YES+KCl

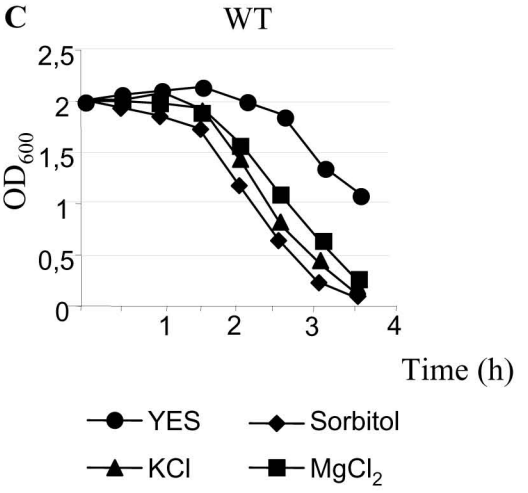

C
WT
2,5
2
1,5
1
0,5
0
OD600
1
2
3
4
Time (h)
YES
Sorbitol
KCl
MgCl2

FIGURE 5

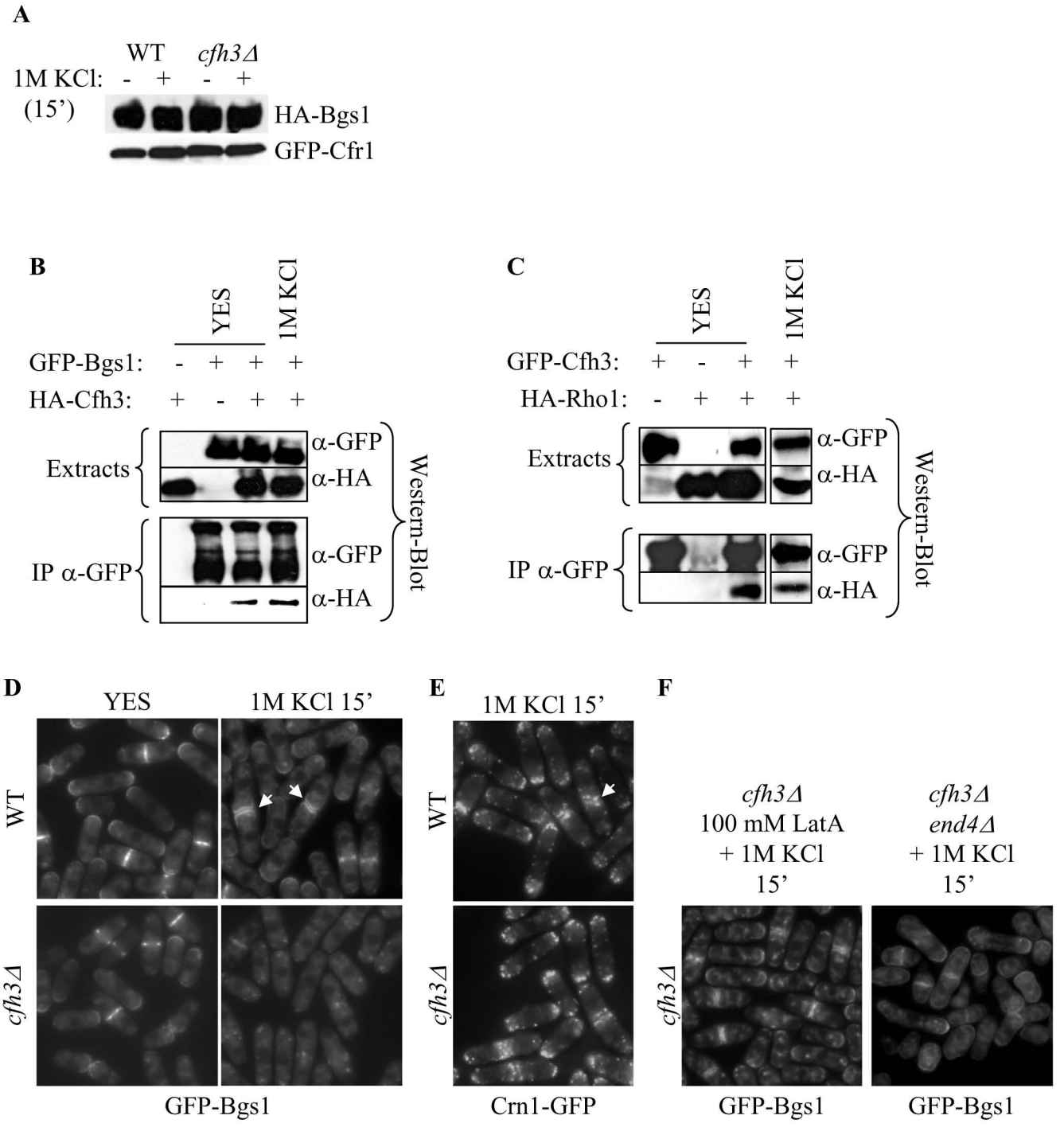
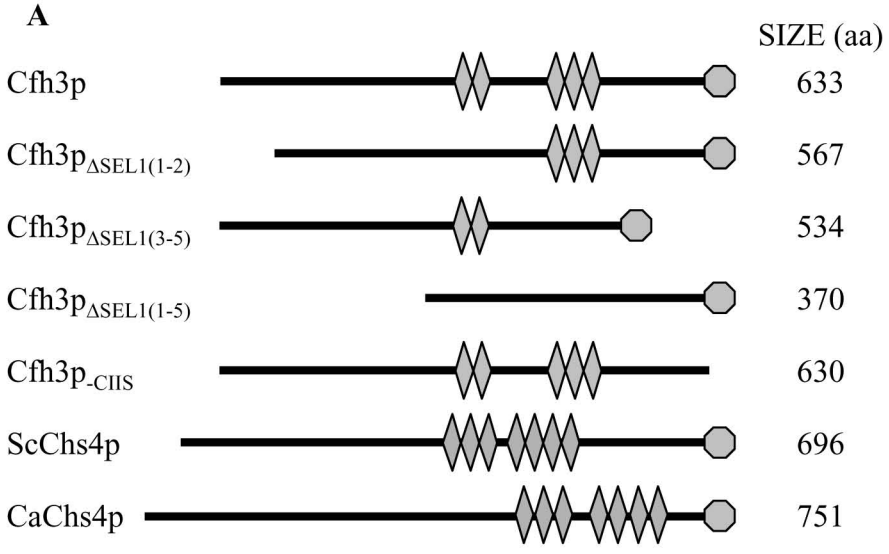
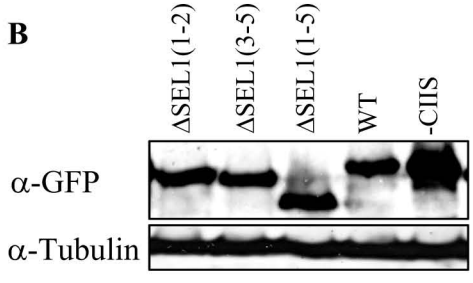
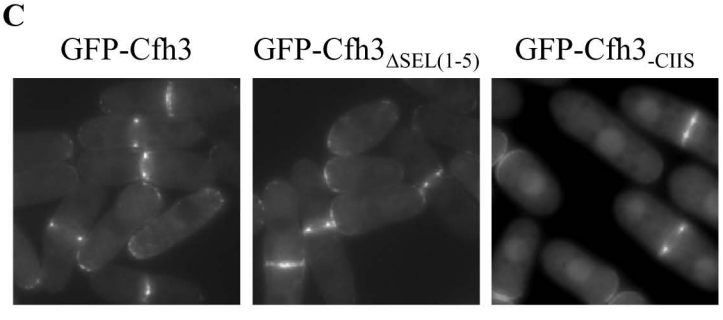
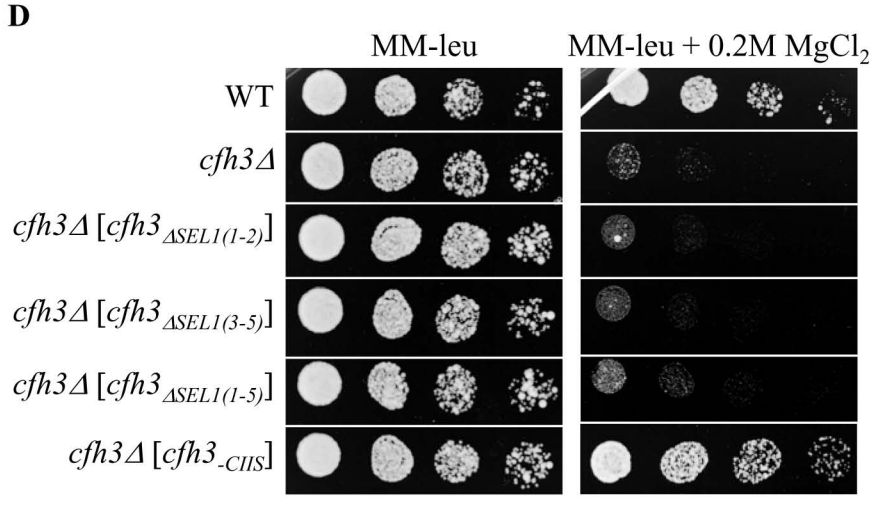

A
WT
cfh3Δ
1M KCl:
(15')
HA-Bgs1
GFP-Cfr1
B
YES
1M KCl
GFP-Bgs1:
HA-Cfh3:
Extracts
α-GFP
α-HA
IP α-GFP
Western-Blot
C
GFP-Cfh3:
HA-Rho1:
D
YES
1M KCl 15'
WT
cfh3Δ
GFP-Bgs1
E
Crn1-GFP
F
cfh3Δ
100 mM LatA
+ 1M KCl
15'
cfh3Δ
end4Δ
+ 1M KCl
15'

FIGURE 6


A
SIZE (aa)
Cfh3p
633
Cfh3p ΔSEL1(1-2)
567
Cfh3p ΔSEL1(3-5)
534
Cfh3p ΔSEL1(1-5)
370
Cfh3p -CIIS
630
ScChs4p
696
CaChs4p
751


B
ΔSEL1(1-2)
ΔSEL1(3-5)
ΔSEL1(1-5)
WT
-CIIS
α-GFP
α-Tubulin


C
GFP-Cfh3
GFP-Cfh3 ΔSEL(1-5)
GFP-Cfh3 -CIIS


D
MM-leu
MM-leu + 0.2M MgCl2
WT
cfh3Δ
cfh3Δ [cfh3 ΔSEL1(1-2)]
cfh3Δ [cfh3 ΔSEL1(3-5)]
cfh3Δ [cfh3 ΔSEL1(1-5)]
cfh3Δ [cfh3 -CIIS]