The FN3 and BRCT motifs in the exomer component Chs5p define a conserved module that is necessary and sufficient for its function

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Runing title: FN3/BRCT domains in Chs5p

Abstract

Chs5p is a component of the exomer, a coat complex required to transport the chitin synthase Chs3p from the trans Golgi network to the plasma membrane. Chs5p N-terminal region exhibits Fibronectin type III (FN3) and BRCT domains. FN3 domains are present in proteins that mediate adhesion processes while BRCT domains are involved in DNA repair. Several fungi -including *Schizosaccharomyces pombe*, which has no detectable amounts of chitin-, have proteins similar to Chs5p. Here we show that the FN3 and BRCT motifs in Chs5p behave as a module that is necessary and sufficient for Chs5p localization and for cargo delivery. The N-terminal regions of *S. cerevisiae* Chs5p and *S. pombe* Cfr1p are interchangeable in terms of Golgi localization but not in terms of exomer assembly, showing that the conserved function of this module is protein retention in this organelle, and that the interaction between the exomer components is organism-specific.

Keywords: Yeast, cell wall, chitin, Golgi, vesicular trafficking.

Abbreviations: TGN, Trans-Golgi network; FN3, Fibronectin type III; BRCT, Breast cancer susceptibility protein C-terminal domain; ChAps, Chs5p-Arf1p-binding proteins; GFP, Green fluorescent protein; RFP, Red fluorescent protein.

Introduction

One of the least studied steps of secretion is protein trafficking between the trans-Golgi network (TGN) and the cell surface. The chitin synthase Chs3p, which produces chitin at the neck between mother and daughter cells and the lateral cell wall [1,2], is a valuable model to investigate vesicular traffic. Chs3p is synthesized constitutively at the endoplasmic reticulum, its exit from this organelle being facilitated by the specific chaperone Chs7p [3,4]. In the TGN, Chs3p is loaded in a subset of secretory vesicles coated by a protein complex termed the exomer ([5-7]; see below) and is delivered to the cell surface. Chs3p localizes predominantly to the mother-bud neck; there, Chs4p anchors the chitin synthase to the septin ring, activates it biochemically, and modulates its endocytic turnover [8-12]. After endocytosis Chs3p is not degraded in the vacuole; instead, it remains in a subpopulation of internal stores/endosomes termed chitosomes [13], from where it can be recycled to become reincorporated in the plasma membrane. Chs3p is one of the best-characterized cargoes that cycle between the plasma membrane and endosomes.

The term exomer refers to a *S. cerevisie* complex of proteins that form a vesicle coat required for the transport of certain proteins from the Golgi apparatus to the cell surface [7]. The exomer is composed of Chs5p, Chs6p, Bch1p, Bch2p and Bud7p [5-7]. Chs5p acts as a scaffold to which Chs6p, Bch1p, Bch2p and Bud7p (termed generically the ChAPs, from Chs5p-Arf1p-binding proteins) bind. All the exomer proteins localize in the TGN [5,6]. The cargo-specificity is provided by the ChAPs; deletion of *CHS6* leads to a defect in chitin synthesis because of a defect in the transport of Chs3p to the plasma membrane [14,15]; deletion of *BUD7* leads to a defect in polarity [16], and deletion of

BCH1 and *BUD7* leads to a mating defect due to a missorting of Fus1p [17]. The role of *BCH2* has not been established because in the analyses performed *bch2Δ* mutants showed no evident phenotype [5,6]. Some ChAPs could have redundant roles for cargo selection since a *bch1Δ bud7Δ* strain phenocopies a *chs6Δ* mutant in terms of its defect in chitin synthesis [5,6]. According to its role as a scaffold for the binding of the ChAPs to the TGN vesicles, deletion of Chs5p results in disruption of the complex, and *chs5Δ* mutants show defects in chitin synthesis, the budding pattern, and mating [18,19]. *chs5Δ* mutants show additional phenotypes, some of which might be due to their defect in the transport of Crh2p, a transglycosylase involved in cell wall synthesis [20].

Chs5 N-terminal region contains two contiguous domains, FN3 and BRCT (see figure 1), which are only 1 to 5 residues apart (depending on the tool used to perform the motif search). FN3 domains are present in proteins involved in cell-surface binding processes and their presence is rare in yeast [21]. They are protein regions of approximately 100 amino acids that exhibit a common tertiary structure but share little primary sequence similarity. They have seven β strands forming a sandwich of two antiparallel β sheets (one containing three strands termed A, B, and E, and the other containing four strands termed C', C, F, and G; [21,22]). The alignment of FN3 motifs found in different proteins (SMART database; [23]) allows the identification of a few conserved residues, which are present in *S. cerevisiae* Chs5p (figure 1) and in *S. pombe* Cfr1p (a protein with a structure similar to that of Chs5p; [24]). Downstream from the FN3 domain there is a BRCT domain (figure 1). BRCT are regions of 85-95 amino acids, characterized by their tertiary structure, that mediate protein-protein interactions and participate in DNA repair and in checkpoints [25,26]. They comprise four β strands forming a β sheet

surrounded by three α helices. The alignment of BRCT motifs in the SMART database leads to the identification of a few conserved residues that are present in most of these domains. Analysis of the three-dimensional structure predicts that some of the conserved residues cannot be altered without perturbing the functionality of the BRCT domains. In particular, a tryptophan located in helix α3, and a glycine located in a loop/turn connecting α1 and β2 are predicted to be important for the structure of the motif [25,26]. Other residues relevant for the functionality of BRCT domains have been identified in analyses of cancer-associated mutations [25,27].

In this work we analyzed the role of the FN3 and BRCT domains in the exomer component Chs5p. We found that both motifs are required for Chs5p localization to the Golgi, and that a truncated protein bearing only these domains is able to localize properly and to support Chs3p delivery to the cell surface. Mutation of some of the conserved residues in the FN3 and BRCT domains reduces Chs5p functionality. Finally, the region of Cfr1p containing the FN3 and BRCT domains localizes to the Golgi in both the fission and the budding yeast, showing that a conserved function of this module is to localize proteins to the Golgi. However, the *S. pombe* truncated protein is not able to support exomer assembly, and consequently Chs3p delivery to the cell surface, showing that the interaction between the exomer components is specific for each organism.

Materials and Methods

Strains and growth conditions

The yeast strains used in this study are derivatives of the α-131-20, L-839 [18] and W303-1A strains. Cells were grown in batches at 32°C in Difco yeast nitrogen base without amino acids (YNB-aa) 6.7 g/litter, containing 2% glucose and the required supplements, or in YEPD (1% yeast extract, 2% Bacto Peptone, 2% dextrose). For solid media, agar was added at 2%. Calcofluor (Blankophor. Bayer) resistance was tested by a plate assay on SD without uracil (SD-URA) medium buffered with 50 mM potassium biphthalate, pH 6.2, as described [18].

Molecular and genetic manipulations

Plasmid KS+CHS5(SmaIATG NotISTOP), carrying a *Sma*I restriction site before the initial ATG and a *Not*I site before the stop codon, was used as a template to perform site-directed mutagenesis with a previously described method [28]. DNA sequencing was used to confirm the accuracy of the sequence in the alleles constructed. Then, the mutated DNA fragments were cloned into a modified pRS316 vector [29] lacking the *Not*I restriction site of the polylinker. To assess the level and localization of the mutated proteins, the HA epitope or the GFP were cloned as *Not*I DNA fragments into the *Not*I site at the C-terminal end of the protein. Deletions inside the FN3 and BRCT domains were produced as follows; *Cla*I sites were introduced between the codons coding for proline at amino acid position 124 and for asparagine at position 125, and between the codons for tyrosine at position 143 and for glutamic acid at position 144. Digestion with *Cla*I and plasmid religation produced the $chs5_{\Delta FN3i}$ allele. The $chs5_{\Delta BRCTi}$ allele was produced by digestion with the *Mlu*I enzyme and religation of a plasmid in which *Mlu*I restriction sites had been introduced between the codons for leucine at position 196 and for serine at position 197, and between the codons for asparagine at position 235 and for asparagine at position 236. To obtain the $chs5_{W98C}$ mutant, the GCATGG sequence,

coding for alanine at position 97 and tryptophan at position 98, was changed to GCATGC (which codes for alanine and cysteine and is a *Sph*I restriction site). The $chs5_{PY143KL}$ allele was produced by changing the CCATAC sequence, coding for proline and tyrosine at positions 142 and 143, respectively, by AAGCTT, coding for lysine and leucine and susceptible to digestion by *Hin*dIII. The $chs5_{W244H}$ mutant was obtained by replacing the TGGGTG sequence (coding for tryptophan at position 244 and for valine at position 245) by CACGTG, which codes for histidine and valine and is susceptible to digestion by *Pma*CI. The $chs5_{GA200TR}$ allele was produced by replacing the GGGGCG sequence, coding for glycine and alanine at positions 200 and 201, respectively, by ACGCGT, coding for threonine and arginine and susceptible to digestion by *Mlu*I. A truncated $Chs5_{\Delta CT}$ protein, lacking the two C-terminal thirds of the protein, was produced by digestion with *Not*I and religation of the KS+CHS5(SmaIATG NotI1009 NotISTOP) plasmid, in which a *Not*I restriction site was inserted after the codon for the phenylalanine at position 274, which is 23 amino acids downstream from the BRCT domain. A $Cfr1_{\Delta CT}$ truncated protein was constructed by digesting with *Not*I and religating a plasmid bearing a $cfr1^{+}$ gene in which *Not*I sites had been introduced 60 bp downstream from the BRCT domain and before the stop codon. A plasmid expressing the Cfr1p FN3 and BRCT domains in *S. cerevisiae* was constructed by replacing the *CHS5* ORF by a *Sma*I/*Not*I DNA fragment coding for the Cfr1p N-terminal end. This plasmid was used as a backbone to construct a plasmid expressing a Chs5:Cfr1 chimeric protein by cloning the *CHS5* sequence coding for the C-terminal end of the protein as a *Not*I/*Not*I DNA fragment.

Protein techniques

Western blotting was performed as described [30]. 4-12% gradient polyacrylamide gels and MES SDS buffer (NuPAGE, Invitrogen) were used to perform the electrophoresis. The α-HA (12CA5; Roche) and α-Tubulin (cloneB-5-1-2. SIGMA) antibodies were used at 1:5000 dilutions.

Microscopy

Images were captured with a Leica DM RXA microscope equipped with a Photometrics Sensys CCD camera, using the Qfish 2.3 program, and processed using the Adobe 7.0 program. To analyze the localization of the sites of active chitin synthesis, cells were incubated in SD-URA medium, buffered with 50 mM potassium biphthalate at pH 6.2, for three hours in the presence of 50 μg/ml of Calcofluor.

Chitin measurement

The amount of chitin was determined in at least three independent experiments as described previously [31].

Brefeldin A treatment

Cells were treated with 25 μg/ml Brefeldin A (SIGMA B7651) for 15 minutes at 28ºC.

Results

The FN3 and BRCT domains are necessary and sufficient for Chs5p localization in the Golgi.

Different motif-searching programs detected the presence of FN3 and BRCT domains in the exomer component Chs5p (see figures 1 and 2 A). The presence of these domains in a Golgi protein involved in cell wall synthesis is intriguing, and we were prompted to

uncover the function that each of the domains might play in the protein. Taking into account that tertiary structure is essential for the functionality of FN3 and BRCT domains, and that there is virtually no separation between these domains in Chs5p (figure 1), it was possible that eliminating one domain would interfere with the structure of the other. In order to overcome this problem, we produced mutant proteins in which 19 amino acid internal to the FN3 domain ($Chs5_{\Delta FN3i}$; Materials and methods and Figure 2 A) or 40 amino acids internal to the BRCT domain ($Chs5_{\Delta BRCTi}$; Materials and methods and Figure 2 A) were deleted. These deletions are 53 amino acids apart from each other. Additionally, we produced a truncated protein ($Chs5_{\Delta CT}$; Material and methods and figure 2 A) lacking the 397 C-terminal amino acids. Western blot analyses showed that all the truncated proteins were expressed in the cells (figure 2 B). Observation of the GFP-fused control and truncated Chs5 proteins under a fluorescence microscope revealed that the $Chs5_{\Delta FN3i}$ and the $Chs5_{\Delta BRCTi}$ proteins were dispersed throughout the cytoplasm in 100% of the cells, while the localization of the $Chs5_{\Delta CT}$ protein was similar to that of Chs5p (figure 2 C). These results were obtained using the W303 and α-131-20 genetic backgrounds (figure 2 and results not shown). Sec7p is a Golgi-resident guanine nucleotide exchange factor for the Arf1 GTPase. Observation of an RFP-tagged Sec7 protein [32] in cells expressing GFP-fused Chs5 proteins showed that the internal deletions of the domains had an effect on Chs5p localization, but did not produce a general alteration in the TGN (central-row panels in figure 2 D and results not shown). Additionally, co-localization analyses confirmed that the presence of the FN3 and BRCT domains together was sufficient for protein localization at the Golgi (88% of RFP-Sec7 dots co-localized with Chs5-GFP dots, n=268; 84% of RFP-Sec7 dots co-localized with $Chs5_{\Delta CT}$-GFP dots, n=209). In order to determine whether the localization of the $Chs5_{\Delta CT}$-GFP protein in the Golgi was direct or mediated by its

interaction with the ChAps, we analyzed its distribution in a ΔΔΔΔChAps strain, deleted for *CHS6*, *BUD7*, *BCH1* and *BCH2* [6]. We found that both Chs5p and Chs5$_{\Delta CT}$p exhibited a discrete localization as cytoplasmic dots in more than 90% of the cells, showing that the truncated protein localized to the Golgi even in the absence of the ChAps (figure 2 E, upper panels). It is known that Arf1-dependent GTPase activity is required for Chs5p localization to the Golgi [6,7]. In order to determine whether Chs5$_{\Delta CT}$ also depended on this activity for its localization, we have used Brefeldin A, an inhibitor of the GTP-exchange factors for Arf1p. Although the use of this drug has the caveat that the Golgi itself disperses with this treatment, Wang *et al*. [7] used it to show the dependence of the exomer assembly on Arf1p, and Trawtein *et al.* [6] used it to show that Chs5p and the ChAPs depend on Arf1p-GTP for Golgi localization. In more than 70% of the cells, both Chs5p and Chs5$_{\Delta CT}$p were dispersed throughout the cytoplasm (figure 2 E, lower panels). The rest of the cells exhibited exaggerated Golgi structures (supplemental material, figure S1) that were not observed after 60 minutes of incubation in the presence of the drug. These results strongly suggested that the full-length and the C-terminal truncated Chs5 proteins had the same requirements for their localization, and confirmed that the presence of the FN3 and BRCT domains was sufficient for Chs5p localization in the Golgi.

The FN3 and BRCT domains are necessary and sufficient for chitin synthesis and Chs3p delivery to the cell surface.

Wild-type budding yeast strains are sensitive to Calcofluor and exhibit defective growth in media supplemented with this chitin-binding dye [33]. However, strains with a defect in the Chs3p-dependent chitin synthesis pathway, such as *chs3Δ*, *chs5Δ* and *chs6Δ*, are resistant to Calcofluor [14]. We tested the capacity of the mutant Chs5 proteins to

complement the resistance to Calcofluor exhibited by the *chs5Δ* mutant [18]. Sensitivity to Calcofluor would show that the altered protein was functional while resistance would show that it was not. We found that, in agreement with its altered localization, neither the $Chs5_{\Delta FN3i}$ nor the $Chs5_{\Delta BRCTi}$ proteins were able to complement the *chs5Δ* mutant (figure 3 A). In contrast, the $Chs5_{\Delta CT}$ protein was able to complement the resistance to Calcofluor of the *chs5Δ* strain (figure 3A). The same result was obtained in the W303, α-131-20 and L-839 genetic backgrounds, although in the W303 background higher Calcofluor concentrations (up to 1 mg/ml) were needed to observe the complementation of the *chs5Δ* mutant by the plasmid bearing the *CHS5* gene (not shown). In order to obtain more accurate information about the functionality of the truncated proteins, we measured the amount of chitin in the same strains. We found that the level of chitin in cells bearing the $Chs5_{\Delta FN3i}$ or the $Chs5_{\Delta BRCTi}$ proteins was as low as that found in cells bearing an empty plasmid, while the level of the polymer in cells bearing the control Chs5 or the truncated $Chs5_{\Delta CT}$ proteins was similar (figure 3 B). In some mutants, chitin is abnormally distributed when the synthesis of this polymer is stimulated by incubating the cells with low amounts of Calcofluor [34]. We analyzed the distribution of the sites of active chitin synthesis by incubating the cells in the presence of a sublethal concentration of Calcofluor (50 μg/ml). As shown in figure 3 C, the *chs5Δ* cells transformed with an empty plasmid or with plasmids expressing the $Chs5_{\Delta FN3i}$ or the $Chs5_{\Delta BRCTi}$ proteins only showed a weak staining around the cells. In contrast, *chs5Δ* cells expressing Chs5p or $Chs5_{\Delta CT}$p from centromeric plasmids exhibited strong fluorescence; in both cases this fluorescence was delimited to the neck between the mother and daughter cells. The same results were obtained in the W303, α-131-20 and L-839 genetic backgrounds (figure 3 C and results not shown). These results showed

that the $Chs5_{\Delta FN3i}$ and the $Chs5_{\Delta BRCTi}$ proteins were not able to promote chitin synthesis in response to the Calcofluor treatment, while $Chs5_{\Delta CT}$ was able to do so. Since Chs5p is an exomer component, and since the exomer is involved in the transport of Chs3p to the membrane, we transformed the strains under study with a centromeric plasmid bearing a GFP-tagged Chs3 protein. We observed that Chs3p was retained in internal vesicles in 100% of the *chs5Δ* cells bearing the empty plasmid and the plasmids expressing the $Chs5_{\Delta FN3i}$ and the $Chs5_{\Delta BRCTi}$ proteins (n=100-150; panels in figure 3D show a cell with the most representative localization for each strain). The distribution of Chs3-GFP in cells expressing Chs5p and $Chs5_{\Delta CT}$p was similar; in the case of cells bearing the pRS316Chs5 plasmid, Chs3p was observed at the mother-bud neck in 79% of the budded cells, in internal vesicles in 19% of the cells, and in both vesicles and the mother-bud neck in 2% of the cells (n=126; figure 3D). In cells expressing the $Chs5_{\Delta CT}$ truncated protein, Chs3p was observed at the mother-bud neck (75% of the cells, n=108; figure 3D), in internal vesicles (22% of the cells) and in the neck and internal vesicles (3% of the cells). Since Chs5p is also required for the transport of Fus1p (a protein required for cell fusion during mating) and some unknown cargo required for polarity, we analyzed the capacity of the mutated Chs5 proteins to complement the mating and polarity defects of the *chs5Δ* mutant. We found that the ratios of trilobated to bilobated zygotes were 0.29 ± 0.02, 1.90 ± 0.1, 0.25 ± 0.02, 0,3 ± 0.03, and 1.95± 0.1 for the *chs5Δ* mutant transformed with the empty vector or the vector expressing the Chs5, $Chs5_{\Delta FN3i}$, $Chs5_{\Delta BRCTi}$ and $Chs5_{\Delta CT}$ proteins, respectively. Additionally, Calcofluor staining allowed us to observe that the budding pattern was axial for the *chs5Δ* mutant transformed with the plasmid expressing the Chs5 and $Chs5_{\Delta CT}$ proteins (supplemental material, figure S2). These results show that, at least for the assays performed and

under the experimental conditions used, the $Chs5_{\Delta CT}$ truncated protein was functional, while Chs5p with deletions in the FN3 or the BRCT domains was not.

Conserved residues in the FN3 and BRCT domains contribute to the localization/functionality of Chs5p

In order to analyze whether some of the conserved residues that are present in the FN3 and BRCT domains are relevant for the localization/function of Chs5p, we mutated the tryptophan at amino acid position 98 (in the FN3 domain; $Chs5_{W98C}$ protein) and/or the tryptophan at position 244 (in the BRCT domain; $Chs5_{W244H}$ and $Chs5_{W98C\ W244H}$ proteins), the proline and tyrosine at positions 142 and 143 (FN3 domain), and the glycine at position 200 and the alanine at position 201 (BRCT domain; $Chs5_{PY143KL\ GA200TR}$ protein), or all six amino acids ($Chs5_{W98C\ W244H\ PY143KL\ GA200TR}$ protein. See materials and methods and the figure 1). Initially, we analyzed the functionality of the mutated proteins by assessing their capacity to confer Calcofluor sensitivity to a *chs5Δ* mutant when expressed in centromeric plasmids. As shown in figure 4 A, the degree of complementation conferred by the different mutated proteins was in the following order: the $Chs5_{W98C}$ protein was the most functional, followed by the $Chs5_{PY143KL\ GA200TR}$ protein, the $Chs5_{W244H}$ protein, the $Chs5_{W98C\ W244H}$ protein, and the $Chs5_{W98C\ W244H\ PY143KL\ GA200TR}$ protein, in whose presence the cells were almost as resistant to Calcofluor as the *chs5Δ* mutant transformed with an empty plasmid. Measurement of the chitin content confirmed that the level of resistance to Calcofluor was correlated with a decrease in the amount of this polymer in the cell wall of the strains under study (figure 4B and results not shown). Fluorescence microscopy analysis of cells bearing the mutated proteins fused to the GFP allowed us to correlate the degree of resistance to Calcofluor with a decrease in the signal observed in the Golgi, and an increase in the

diffuse fluorescence throughout the cytoplasm (figure 4 B and results not shown). We next wished to evaluate the capacity of the mutated proteins to allow exomer assembly by observing the distribution of a GFP-fused Chs6 protein. We were unable to detect any specific signal when this fused protein was expressed from a centromeric plasmid in a WT strain (results not shown). When expressed from a multicopy plasmid, 30-50% of WT cells (depending on the culture conditions) exhibited a diffuse fluorescence, while 70-50% of the cells exhibited a discrete fluorescence in cytoplasmic dots (results not shown). When this protein was expressed in *chs5Δ* cells bearing centromeric plasmids that expressed different Chs5 proteins, the percentage of cells with discrete fluorescent dots was as follows: 0% in cells bearing the empty pRS316 vector and the $Chs5_{W98C\ W244H\ PY143KL\ GA200TR}$ protein (n=150-200; see figure 4D); 59% in cells expressing Chs5p (n=127), 48% in cells expressing the $Chs5_{W244H}$ protein (n=143), and 44% in cells expressing the $Chs5_{W98C\ W244H}$ protein (n= 194). Finally, we evaluated the capacity of the Chs5 variants to deliver Chs3p to the cell surface by observing a GFP-fused Chs3 protein. When the *chs5Δ* cells bore the pRS316 empty vector or a plasmid expressing the $Chs5_{W98C\ W244H\ PY143KL\ GA200TR}$ mutant protein, 100% of the cells exhibited a discrete fluorescence in cytoplasmic dots (n=150-200; see figure 4 D); when the cells expressed the native Chs5 protein, Chs3p was observed at the mother-bud neck (71% of the cells), in internal vesicles (21% of the cells), and in both vesicles and the mother-bud neck (8% of the cells; n=154. Figure 4D). In *chs5Δ* mutants expressing the $Chs5_{W244H}$ mutant protein, Chs3p was observed at the mother-bud neck (23% of the cells), in internal vesicles (35% of the cells), and in the neck and internal vesicles (42% of the cells; n=190). When the *chs5Δ* cells expressed the $Chs5_{W98C\ W244H}$ mutant protein, Chs3p was observed at the mother-bud neck (16% of the cells), in internal vesicles (41% of the cells), and in the neck and internal vesicles (43% of the cells; n=165). These results

showed that a decreased capacity of the Chs5 mutant proteins to localize in the Golgi was correlated with a low capacity to assemble the exomer, to deliver Chs3p to the cell surface and, consequently, to support chitin synthesis. Additionally, these results confirmed that both domains participate in the localization of Chs5p in the TGN, and that small alterations in these domains cause a decrease in Chs5p functionality.

The Cfr1p FN3 and BRCT domains are sufficient to localize the protein to the Golgi in both Schizosaccharomyces pombe and Saccharomyces cerevisiae

Cfr1p is a *S. pombe* protein required for mating that contains FN3 and BRCT domains in its N-terminal region [24]. In order to determine whether the FN3 and BRCT domains in the *S. pombe* Cfr1 protein were sufficient to localize it to the Golgi, as it occurs with the budding yeast Chs5p, we constructed a GFP-tagged Cfr1 protein lacking its C-terminal end (see Materials and methods and figure 5 A). We found that in *S. pombe* the localization of the truncated and the full-length protein was similar (figure 5 B). Next, we expressed in *S. cerevisiae* the GFP-fused Cfr1$_{\Delta CT}$ truncated protein and a Cfr1:Chs5 chimeric protein in which the N-terminal region of Cfr1p was fused to the C-terminal region of Chs5p (see Materials and methods and figure 5 A). When expressed in the budding yeast, most of the signal corresponding to both GFP-fused Cfr1$_{\Delta CT}$ and Cfr1:Chs5 proteins localized as florescent dots in the cytoplasm, although some diffuse fluorescence was observed in the cytoplasm (figure 5 C). Co-localization of the green dots with the TGN marker Sec7p fused to the RFP occurred in 88% of the dots (n=173) in *chs5Δ* cells expressing the Chs5-GFP protein, in 81% of the dots (n=139) in *chs5Δ* cells expressing the Cfr1$_{\Delta CT}$-GFP protein, and in 83% of the dots (n=123) in *chs5Δ* cells expressing the Cfr1:Chs5-GFP protein (figure 5 D), confirming that the proteins containing the Cfr1p FN3 and BRCT domains localized to the Golgi.

The Cfr1p FN3 and BRCT domains cannot regulate chitin synthesis in Saccharomyces cerevisiae

Upon finding that the FN3 and BRCT domains of Cfr1p were able to localize to the Golgi in *S. cerevisiae*, we wondered whether they were functional in the budding yeast; i.e., whether they were able to support exomer assembly and to regulate chitin synthesis. In order to address this question, we analyzed the capacity of the $Cfr1_{\Delta CT}$ and Cfr1:Chs5 proteins to complement the defects of null *chs5Δ* mutants. We found that *chs5Δ* strains (in the W303 and the α-131-20 genetic backgrounds) bearing an empty vector or plasmids that expressed the $Cfr1_{\Delta CT}$ or Cfr1:Chs5 proteins behaved similarly in terms of Calcofluor resistance (figure 6 A) and chitin synthesis (figures 6 B and 6 C). These results showed that the N-terminal regions of Chs5p and Cfr1p are not interchangeable for chitin synthesis. In order to determine the reason for the inability of the Cfr1p N-terminal region to regulate this process, we analyzed the distribution of the exomer component Chs6p (fused to the GFP and expressed from a multicopy plasmid) and the chitin synthase Chs3p (fused to the GFP and expressed from a centromeric plasmid) in a *chs5Δ* strain transformed with an empty vector and the plasmids expressing the Chs5, $Chs5_{\Delta CT}$, $Cfr1_{\Delta CT}$, and Cfr1:Chs5 proteins. In the mutant strains bearing the empty plasmid, the fluorescence of the Chs6-GFP protein was dispersed throughout the cytoplasm and that of the GFP-Chs3 protein was observed in internal vesicles in 100% of the cells (n=100-150; figure 6D). When the *chs5Δ* mutant cells expressed the full-length Chs5 protein, Chs6p was observed as discrete dots in the cytoplasm in 53% of the cells (n=180) and Chs3p was observed at the mother-bud neck in 76% of the cells, in internal vesicles in 18% of the cells, and in both vesicles and the mother-bud neck in 6% of the cells (n=174; figure 6 D). When the *chs5Δ* mutant cells expressed the

Chs5$_{\Delta CT}$ protein, Chs6p was observed as discrete dots in the cytoplasm in 53% of the cells (n=210) and Chs3p was observed at the mother-bud neck (75% of the cells), in internal vesicles (20% of the cells), and in the neck and internal vesicles (5% of the cells; n=166). When the Cfr1$_{\Delta CT}$ or the Cfr1:Chs5 proteins were expressed in the *chs5Δ* mutant, Chs6p was dispersed throughout the cytoplasm and, accordingly, Chs3p could not reach the cell surface in 100% of the cells (n=150-180; figure 6 D and results not shown). These results showed that the N-terminal region of Cfr1p was not able to promote chitin synthesis because it was not able to ensure Chs6p localization in the Golgi, and strongly suggested that the interaction between the scaffold and the other exomer components is organism-specific.

Discussion

Vesicular transport is a major cellular activity that ensures protein trafficking between specific membrane-enclosed compartments. Protein transport from the ER to/from the cis- or intermediate-Golgi compartments has been subjected to detailed analysis ([35]; for a review see the FEBS letters special issue on the Golgi apparatus. Volume 583). Coat proteins participate in most of the vesicular traffic events in the cell and contribute to cargo-specificity. The TGN is viewed as a central station from which proteins are sorted and targeted to their final destination. One of the least well understood steps of secretion is the trafficking between the TGN and the plasma membrane. The exomer is a protein complex that forms a coat structure required for the transport of certain proteins from the TGN to the cell surface [5-7,17,18,19,20,36]. In this work we performed a structure-function analysis of Chs5p, a Golgi protein that is essential for the integrity of the complex and for the localization of the ChAps in the TGN [5-7]. Chs5p lacks all known sequences for retention at the Golgi apparatus and the

bioinformatic tools predict that it is a nuclear protein. It has been shown that *E. coli*-expressed Chs5p C-terminal end is able to interact with lipids in an overlay assay using phosphoinositide strips [7], suggesting that this part of the protein could mediate the recruitment of the protein to the Golgi membrane. Thus, it was expected that the N-terminal end of the protein, containing the FN3 and BRCT domains, would be responsible for the interaction with the ChAps and/or cargo proteins. In agreement with this notion, Chs5 amino acids 1-260 interact with Chs6p in a two-hybrid assay [7]. In order to uncover the function that those domains might play in the protein, we eliminated the central amino acids of each of them. We found that both domains had the same function in the protein, being required for Chs5p localization in the Golgi. This result was confirmed by mutating some of the conserved residues in the motifs. Substitution of tryptophan at position 244 (located on the α3 helix, at the centre of a conserved hydrophobic pocket, and considered the hallmark of the BRCT domains) by a histidine had a significant impact on the functionality of the protein. Additional mutations in other conserved residues in the BRCT and FN3 domains reduced Chs5p functionality. These results confirmed that both domains are required for Chs5p localization in the Golgi and that the computer-predicted motifs are *bona fide* FN3 and BRCT domains. The amino-terminal end of the protein (amino acids 1-274) proved to be sufficient to promote protein localization to the TGN and to be functional in terms of exomer assembly and chitin synthesis. This is in agreement with the results of Wang *et al.*, who found that amino acids 1-401 complemented the chitin synthesis defect of a *chs5Δ* strain [7]. The N-terminal region of the protein was able to localize to the TGN even in the absence of the ChAps, ruling out the possibility that these proteins might mediate the localization of the truncated Chs5 protein. Wang *et al.* reported that the C-terminal end of Chs5p was able to interact with lipids in an overlay assay [7] and

suggested that this part of the protein facilitates the membrane recruitment of Chs5p. We have found that Chs5p is recruited to the Golgi even in the absence of its C-terminal end. Thus, either the C-terminal region of Chs5p does not interact with phosphoinositides *in vivo*, or this interaction is not necessary for the localization of the protein to the Golgi. Also, it is possible that the lipid-Chs5p interaction plays some minor role in the functionality of the protein that cannot be detected under laboratory conditions. This does not mean that in nature, where environmental conditions are extreme, this part of the protein might have some relevance. It has been described that Chs5p runs slower than expected in polyacrylamide gels [36]; we found the same result for the $Chs5_{\Delta FN3i}$ and the $Chs5_{\Delta BRCTi}$ truncated proteins, but not for the $Chs5_{\Delta CT}$ protein. Thus, the C-terminal region of the protein seems to undergo significant post-translational modifications. This region of the protein contains the 2 potential O-glycosylation sites; 5 out of the 6 potential N-glycosylation sites; the 2 potential SUMOylation sites, and 36 out of the 44 potential phosphorylation sites predicted by different programs (ExPASy Proteomics Server). However, the biological relevance of these modifications is difficult to establish, since this part of the protein seems to be dispensable for both localization and function.

Our results show that the FN3 and BRCT domains in Chs5p constitute a module that mediates protein localization to the Golgi, exomer assembly, and Chs3p delivery to the membrane. Both domains are required for this function since total deletion (results not shown) or small alterations in each domain abrogate the localization of the protein, exomer assembly, and chitin synthesis. This is a new function for the FN3 and BRCT domains which have been described to have roles in protein-protein interactions in the

extracellular matrix and in DNA remodelling, respectively. One of the few exceptions is Ect2p, a guanine nucleotide exchange factor (GEF) for Rho A, in which the tandem BRCT domains are required for proper function of the protein during cytokinesis [37].

Proteins with consecutive FN3 and BRCT domains have only been found in fungi. With the exception of a hypothetical protein from the basidiomycete *Filobasidiella neoformans*, all other organisms bearing these proteins are ascomycetes. This suggests that that in these organisms these domains appeared as the consequence of a horizontal transmission of genetic information. The fact that in all the proteins bearing these domains the FN3 is always N-terminal with respect to the BRCT domain suggests that this organization is relevant for function; this is in agreement with the hypothesis that both motifs function as a single module, for which the three-dimensional structure provided by each domain is essential. With the exception of the *S. cerevisiae* Chs5 protein (involved in chitin synthesis and mating; [18]) and the *S. pombe* Cfr1 protein (required for mating; [24]) these fungal proteins have not been characterized. We found that the N-terminal region of Cfr1p was sufficient to localize the protein to the TGN in both *S. pombe* and in *S. cerevisiae* efficiently, showing that the conserved function of the module (formed by the FN3 and BRCT domains) is to localize proteins to this cellular organelle. However, although the pattern of localization of the $Cfr1_{\Delta CT}$ or Cfr1:Chs5 proteins is similar to that of the $Chs5_{W244H}$ protein, the *S. pombe* domains are not able to promote exomer assembly or regulate chitin synthesis in *S. cerevisiae*, while the $Chs5_{W244H}$ mutant protein retains a high degree of functionality. These results are in agreement with our previous observation that Chs5p and Cfr1p can be expressed in *S. pombe* and *S. cerevisiae*, respectively, but they are not able to complement the

phenotypes of the corresponding mutants ([24] and our unpublished results). We have found that the reason for the lack of complementation of the chitin synthesis defect in the *S. cerevisiae chs5Δ* mutant by Cfr1p is that the exomer component Chs6p is not able to localize in the Golgi and, therefore, Chs3p is not delivered to the cell surface. These results strongly suggest that the interaction between the scaffold and the other proteins in the exomer complex is specific to each organism. The N-terminal regions of Chs5p and Cfr1p are 46% identical and 63% similar. Probably, the C-terminal region of the ChAps, which is responsible for their interaction with Chs5p [6], interacts with non-conserved residues in the Chs5/Cfr1 N-terminal region.

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Figure legends

Figure 1. Primary structure (upper line) and secondary structure (lower line. E, β strands; H, α helices; C, coil) corresponding to the Chs5p region comprising amino acids 76 to 255. The FN3 or BRCT domains are underlined. Amino acids forming β strands are depicted in red and amino acids corresponding to α helices are in blue. The strands and helices have been named according to the nomenclature established for each domain. The amino acids missing in the $Chs5_{\Delta FN3i}$ and $Chs5_{\Delta BRCTi}$ proteins are double-underlined. Asterisks mark the conserved residues that were mutated in the $Chs5_{W98C}$, $Chs5_{PY143KL}$, $Chs5_{W244H}$ and $Chs5_{GA200TR}$ proteins.

Figure 2. The FN3 and BRCT domains are necessary and sufficient for localization of Chs5p to the Golgi. A. Schematic representation of Chs5p and the indicated mutated proteins. The predicted molecular weight is given in kDa. B. Western blots showing the level of the indicated HA-tagged Chs5 proteins (upper blot) compared to the level of tubulin (lower blot). KDa indicates the size of the proteins in the molecular weight standard. The asterisk marks an unspecific band recognized by the α-HA antibody. C. Fluorescence micrographs of cells carrying the indicated Chs5 proteins fused to the GFP. D. Fluorescence micrographs showing the localization of Sec7-RFP and the indicated GFP-fused Chs5 variants. E. Localization of GFP-fused Chs5 and $Chs5_{\Delta CT}$ proteins in a strain deleted for the four ChAp proteins (upper panels) or in cells treated with the Sec7 inhibitor Brefeldin A (lower panels). Bar, 10 μm.

Figure 3. The FN3 and BRCT domains are necessary and sufficient for the proper regulation of chitin synthesis. The Chs5 proteins used in this figure were untagged. A. $3x10^4$ cells and serial 1:4 dilutions from a *chs5Δ* strain bearing the indicated plasmids were spotted onto buffered SD-URA plates supplemented with the indicated amounts of Calcofluor and incubated for two days at 32ºC. B. Amount of chitin in a *chs5Δ* strain bearing centromeric plasmids producing the indicated Chs5 proteins. C. Distribution of chitin in the same strains as in B incubated in the presence of 50 μg/ml of Calcofluor for three hours. D. Localization of a GFP-fused Chs3 protein in a *chs5*Δ strain bearing the indicated plasmids. Cells in the micrographs show the most representative Chs3p distribution for each strain. Bar, 10 μm.

Figure 4. Conserved residues in the FN3 and BRCT domains are relevant for Chs5p localization and functionality. A. $3x10^4$ cells and serial 1:4 dilutions from a *chs5Δ* strain bearing the indicated plasmids were spotted onto buffered SD-URA plates

supplemented with the indicated amounts of Calcofluor and incubated for two days at 32ºC. The Chs5 proteins were untagged. B. Amount of chitin in a *chs5Δ* strain bearing centromeric plasmids producing the indicated untagged Chs5 proteins. C. Localization of the indicated Chs5 proteins fused to the GFP in *chs5Δ* cells (upper-row panels) or localization of GFP-fused Chs6p (middle-row panels) and GFP-fused Chs3p (lower-row panels) in *chs5Δ* cells expressing the indicated untagged Chs5 proteins from centromeric plasmids. Cells in the micrographs show the most representative distribution of the GFP-fused proteins in each strain. Bar, 10 μm.

Figure 5. The amino terminal end of Cfr1p directs protein localization to the Golgi in both *Schizosaccharomyces pombe* and *Saccharomyces cerevisiae*. A. Schematic representation of *S. cerevisiae* Chs5p, *S. pombe* Cfr1p, a truncated Cfr1 protein missing the C-terminal end of the protein ($Cfr1_{\Delta CT}$), and a chimera bearing the N-terminal region of Cfr1p containing the FN3 and BRCT domains, and the C-terminal region of Chs5p (Cfr1:Chs5). B. Localization of the full-length Cfr1 and the $Cfr1_{\Delta CT}$ truncated proteins, fused to the GFP, in *S. pombe*. C. Localization of the indicated proteins fused to the GFP in a *chs5Δ S. cerevisiae* strain. D. Localization of Sec7-RFP and the C-terminal truncated Cfr1 protein in *S. cerevisiae*; the panel on the right shows a superimposition of the RFP and GFP images. Bar, 10 μm.

Figure 6. The amino terminal region of Cfr1p is not able to promote either chitin synthesis or Chs3p delivery. The Chs5 proteins used in this figure were untagged. A. $3x10^4$ cells and serial 1:4 dilutions from a *chs5Δ* strain bearing the indicated plasmids were spotted onto buffered SD-URA plates supplemented with the indicated amounts of Calcofluor and incubated for two days at 32ºC. B. Amount of chitin in a *chs5Δ* strain bearing centromeric plasmids producing the indicated Chs5 proteins. C. Distribution of

chitin in the same strains as in B, incubated in the presence of 50 μg/ml of Calcofluor for three hours. D. Localization of a GFP-fused Chs6 protein (upper panels) or Chs3 protein (lower panels) in strains bearing the indicated plasmids. Cells in the micrographs show the most representative distribution of the GFP-fused proteins in each strain. Bar, 10 μm.

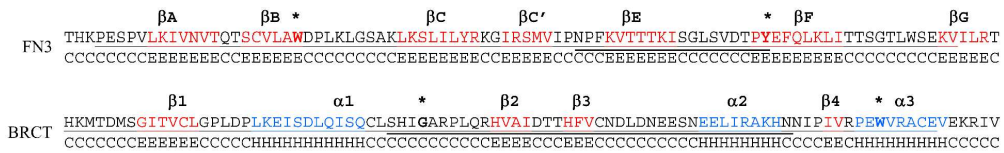

Figure 1
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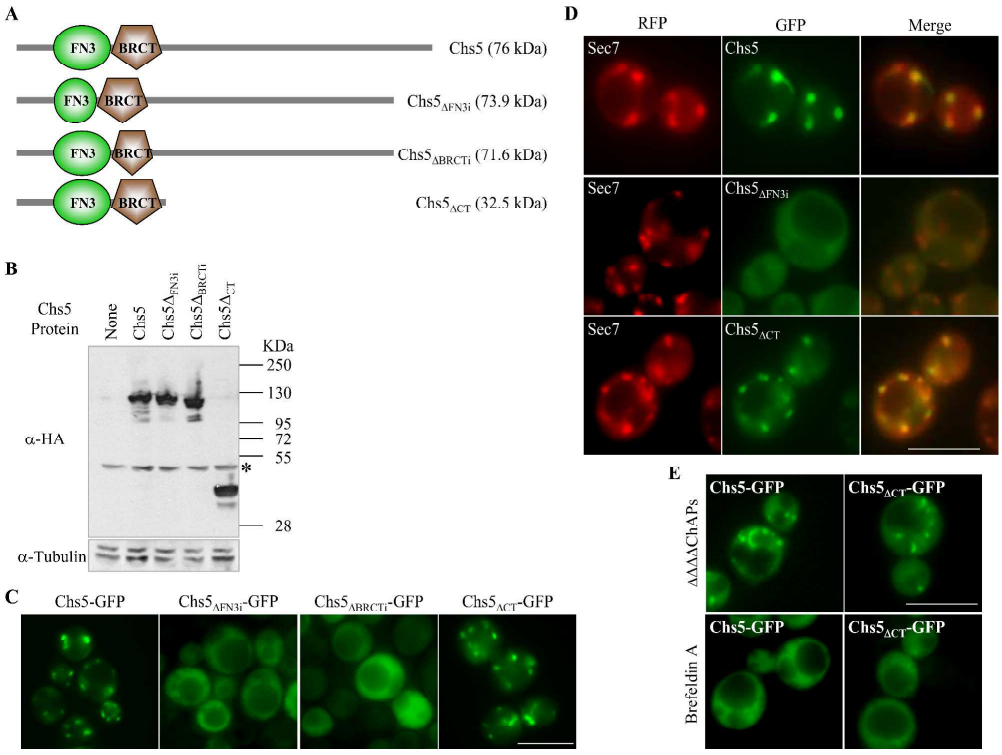

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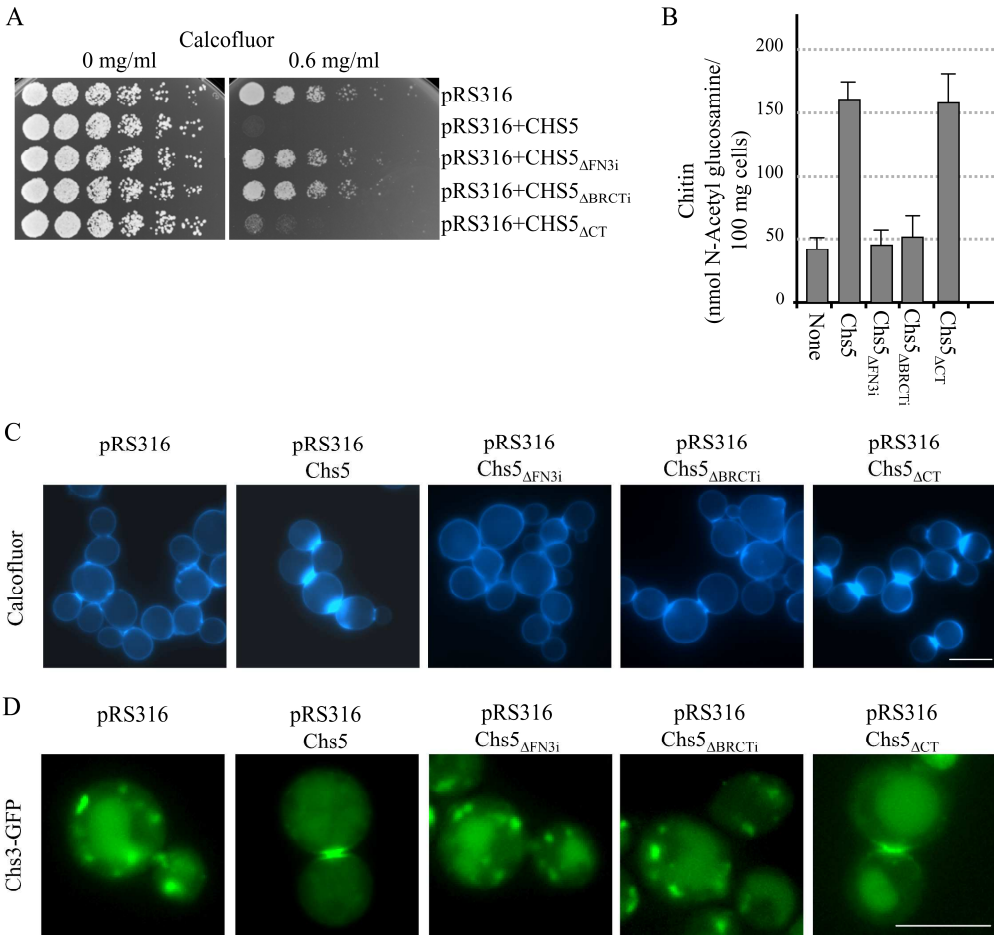

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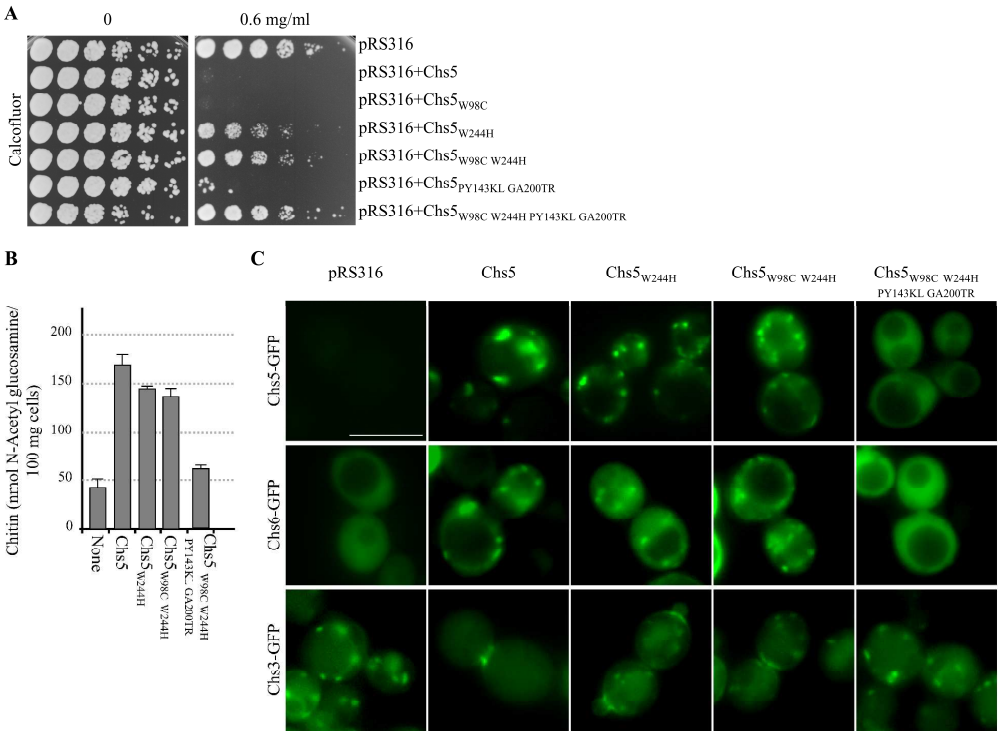

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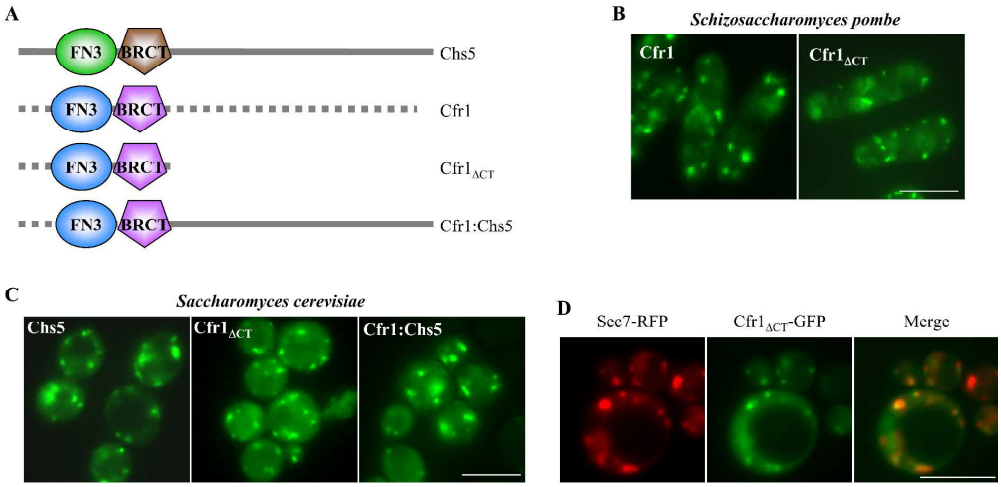

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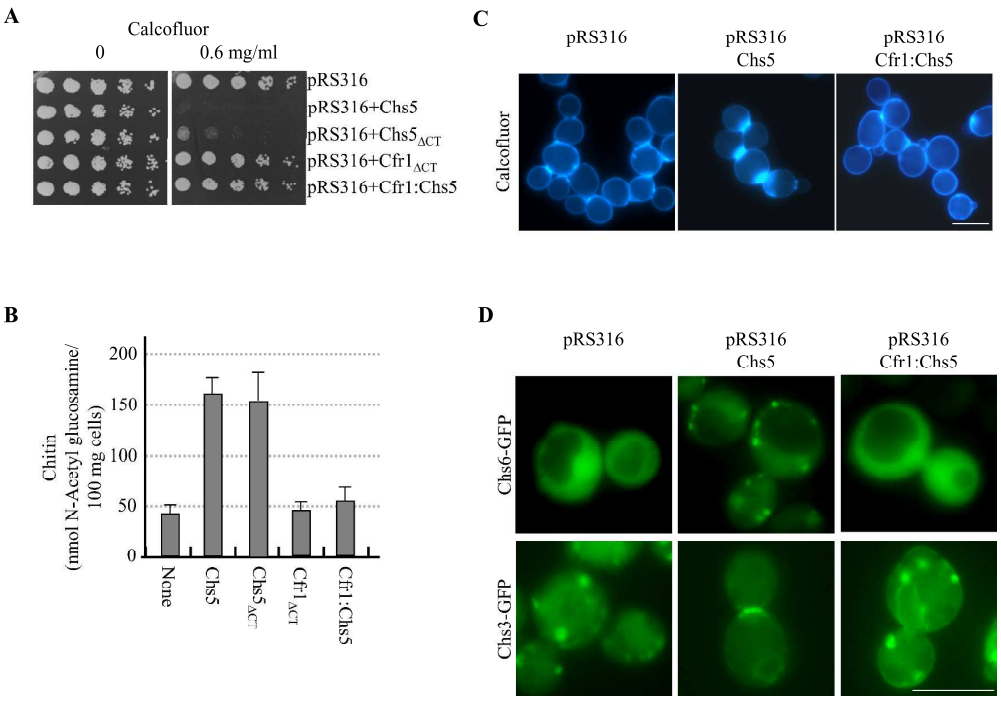

Figure 6
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