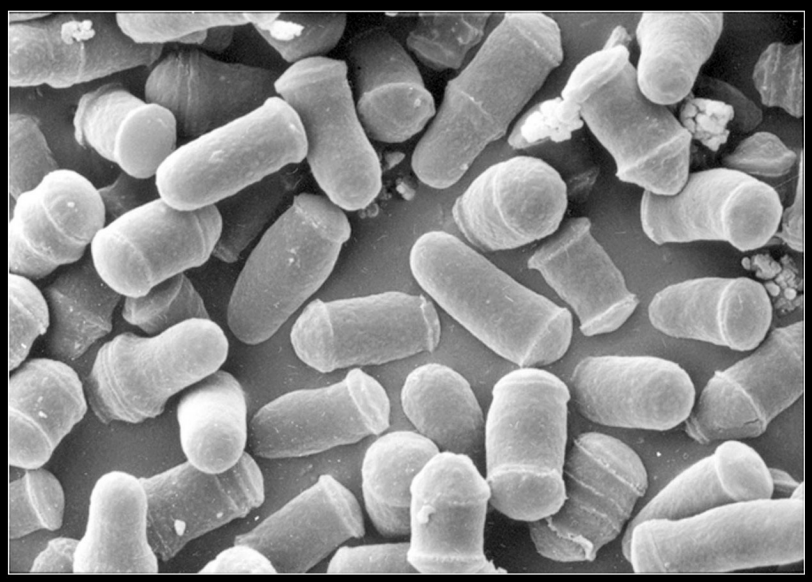
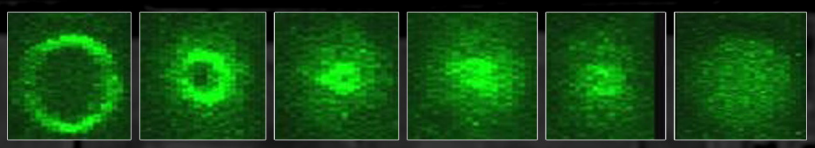
# Role of Cfh3p in the morphogenesis of *Schizosaccharomyces pombe*





Mirza Mohammad Reza SHARIFMOGHADAM

Ph.D. Thesis

2009

Departamento de Microbiología y Genética
Instituto de Microbiología Bioquímica
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**El Dr. D. Ángel Domínguez Olavarri, Director del Departamento de Microbiología y Genética, de la Universidad de Salamanca**

**CERTIFICA:**

Que la memoria titulada "Estudio de la función de Cfh3p en la morfogénesis de *Schizosaccharomyces pombe*", presentada por D. Mirza Mohammad Reza SHARIFMOGHADAM para optar al grado de Doctor en Biología ha sido realizada en Instituto de Microbiología Bioquímica, centro mixto de la Universidad de Salamanca (Departamento de Microbiología y Genética) y el Consejo Superior de Investigaciones Científicas bajo la dirección de la Dra. Henar Valdivieso Montero.

Y AUTORIZA su presentación y evaluación por el tribunal correspondiente, para lo que firma este certificado en Salamanca a 30 de Junio de 2009.

**Dr. Angel Dominguez Olavarri, Director of the Department of Microbiology and Genetics, from the University of Salamanca,**

**CERTIFIES**

That the work entitled "Role of Cfh3p in *Schizosaccharomyces pombe* morphogenesis", presented by Mirza Mohammad Reza SHARIFMOGHADAM to get the Doctoral degree has been developed in the Institute for Biochemical Microbiology/Department of Microbiology and Genetics (University of Salamanca/Spanish Council of Research) under the supervision of Dr. Henar Valdivieso.

I thereby GIVE MY AUTHORIZATION for its defence by the corresponding committee. Signed in Salamanca, the 30 day of June, 2009.

Fdo. Dr. Ángel Domínguez Olavarri.

**La Dra Henar Valdivieso, Profesora Titular del Departamento de Microbiología y Genética de la Universidad de Salamanca**

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I would like to thank all the persons that made this work possible in a way or another.

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*This work is dedicated to*
*Mirza Abbas Sharifmoghadam,*
*a wise man, my Father.*

*To all my Family*

# Abbreviations

| | |
|---|---|
| **ARS** | **A**utonomous **R**eplicative **S**equence |
| **CAR** | **C**ontractile **A**ctomyosin **R**ing |
| **CS** | **C**hitin **S**ynthase |
| **DIC** | **D**ifferential **I**nterference **C**ontrast |
| **DMSO** | **D**i**M**ethyl **S**ulf**O**xide |
| **ECL** | **E**nhanced **C**hemmo**L**uminiscence |
| **EMM** | **E**dinburgh **M**inimal **M**edium |
| **EDTA** | **E**thylene **D**iamine **T**etraacetic **A**cid |
| **GS** | **G**lucan **S**ynthase |
| **GFP** | **G**reen **F**luorescent **P**rotein |
| **GST** | **G**lutathione **S** **T**tansferase |
| **GTPase** | **G**uanosine **T**ri**P**hosphate **(GTP)** hydrolase. |
| **HA** | **H**em**A**glutinine |
| **HA6H** | **H**em**A**glutinine and 6 **H**istidide residues |
| **IP** | **I**mmuno**P**recipitation |
| **IPTG** | **I**sopro**P**yl-beta-D-**T**hio**G**alactopyranoside |
| **LB** | **L**uria-**B**ertani |
| **MAPK** | **M**itogen **A**ctivated **P**rotein **K**inase |
| **MCS** | **M**ulti **C**loning **S**ite |
| **MM** | **M**inimal **M**edium |
| **MT** | **M**icro**t**ubule |
| **MTOC** | **M**icrotubule **O**rganizing **C**entre |
| **O.D.** | **O**ptical **D**ensity |
| **PBS** | **P**hosphate **B**uffered **S**aline |
| **PCR** | **P**olimerase **C**hain **R**eaction |
| **RBD** | **R**ho **B**inding **D**omain |
| **RFP** | **R**ed **F**luorescent **P**rotein |
| **SIN** | **S**eptation **I**nitiation **N**etwork |
| **SPA** | **Sp**orulation **A**gar |
| **TCA** | **T**ri**C**hloroacetic **A**cid |
| **TGN** | **T**rans **G**olgi **N**etwork |
| **ToRFP** | **To**mato **R**ed **F**luorescent **P**rotein |
| **Ts** | **T**hermo **s**ensitive |
| **Vic** | **V**iable in the presence of **I**mmunosupresant and **C**hloride |
| **WT** | **W**ild **T**ype |
| **YES** | **Y**east **E**xtract with **S**upplements |
| **YT** | **Y**east extract with **T**ryptone |

# Index

# Introduction

## 1. The Budding and Fission yeasts as model organisms for the study of cell cycle and morphogenesis

Yeasts were the first microorganisms used by humans. Products obtained through the activity of yeasts have been present in our society for centuries. During the fifties, Herschel Roman proposed *Saccharomyces cerevisiae* as a model organism and, similarly, Murdoch Mitchison recognized *Schizosaccharomyces pombe* as a model system for studying cell division and growth (Mitchison, 1957). Since then, the general knowledge of cell cycle and morphogenesis in eukaryotes has advanced significantly. A series of characteristics make both yeasts interesting model organisms; they are small organisms that need neither large spaces nor culture's special conditions, they are not pathogenic, their generation time is short so that they multiply quickly, they are easy to manipulate in the laboratory and easy to analyze genetically and microscopically, and they have short and well-characterized sexual cycles. Additionally, their genomes were completely sequenced through worldwide collaborations in 1996 and in 2002, respectively (Goffeau *et al.*, 1996, Wood *et al.*, 2002). All these characteristics, together with the development and improvement of the techniques required for their manipulation, have confirmed the potential of both yeasts as model organisms. Cell cycle control is better characterized in yeasts than in any other organism and it is accepted that the data obtained studying *S. cerevisiae* and *S. pombe* can be extrapolated to other eukaryotic organisms. This knowledge can contribute to the understanding of the mechanisms leading to some human diseases such as cancer. Additionally, *S. pombe* has become a useful model organism for studying morphogenesis and cytokinesis because in this yeast, as in higher eukaryotes, this process involves the assembly and contraction of an acto-myosin ring (Nurse, 1994, Yanagida, 2002)

## 2. Life cycle of *Schizosaccharomyces pombe*

The fission yeast *S. pombe* was described and isolated in Africa in 1893 by P. Lindner from a variety of African beer. In 1921, A. Osterwalder isolated from a grape juice in the south of France a homothallic strain that he called $h^{90}$ because about 90% of cells were able to conjugate and form spores in poor media. This strain of *S. pombe* was characterized genetically by U. Leupold in the forties and now it is known as strain 972. Heterothallic $h^+$ 972 and $h^-$ 972 strains were isolated from $h^{90}$ 972 and they gave rise to most strains used nowadays in most laboratories.

*S. pombe* is an ascomycete that, under natural conditions, is haploid. It has 13.8 Mb DNA, which codifies 4824 genes organized in 3 chromosomes. The complete sequence of the genome of this yeast was published in February 2002 (Wood *et al.*, 2002) and is accessible in (www.sanger.ac.uk/Projects/S_pombe). *S. pombe* haploid cells belong to one of two different sexual types, $h^+$ or $h^-$. In normal growth conditions, the cells display a typical eukaryotic cell cycle that includes G1, S, G2 and M phases, although *S. pombe* does not display a very obvious G1 phase due to its short duration.

*S. pombe* cells are cylindrical; newly-born cells have a diameter of 3-4 µm and a length of 7-8 µm. During growth, the cells maintain a constant diameter but increase in length until reaching 12-15 µm, which is the size required for division. Vegetative reproduction takes place through cross-sectional fission, by the formation of a septum perpendicular to the longest axis of the cell. After cytokinesis finishes, this septum is degraded giving rise to two daughter cells of identical size (figure 1, a). *S. pombe* strains used in the laboratory can be continuously maintained in haploid state. Its generation time varies between 2 and 4 hours.

In nutrient-deficiency media cells remain arrested in the G1 phase (if nitrogen is absent) or in the G2 phase (if glucose is absent) of the cell cycle. If cells of different sexual type are incubated in nitrogen-devoid media, they suffer a series of morphogenetic changes that lead to the conjugation process and the formation of a diploid zygote (Egel, 1994). The mating process begins with the formation of a projection in a pole of each mating cell, which is called *shmoo*, until both cells establish contact (figure 1, b) and fuse forming the zygote (figure 1, c). Zygotes are unstable and usually undergo meiosis immediately, which leads to the formation of four haploid nuclei; each one is located inside one haploid spore and these spores are

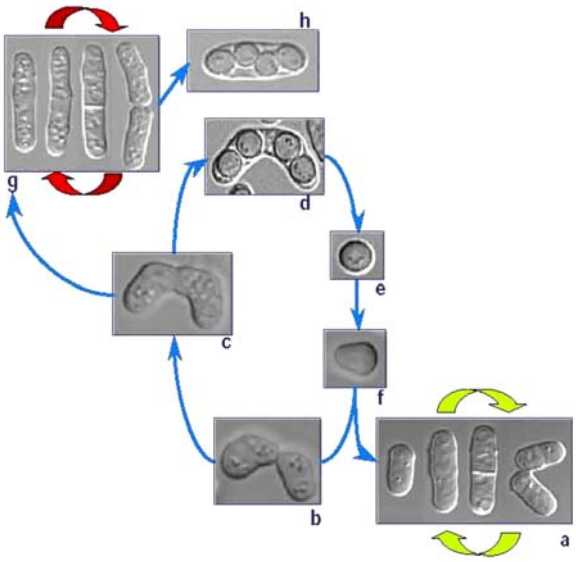



**Figure 1. Life cycle of *Schizosaccharomyces pombe***. a-h represent different moments of the life cycle (see the text for further information).

inside a zygotic ascus (figure 1, d). Each tetrad contains two spores of each sexual type. The separation of these 4 ascospores by the micromanipulation method (tetrads analysis) is the basis for the genetic analysis of this organism. After a time, the wall of the asci breaks releasing the haploid spores (figure 1, e) that will remain latent until they find favorable conditions and germinate (figure 1, f) reinitiating the haploid cycle.

Despite the fact that diploid cells are unstable, they can have a diploid mitotic cycle (Egel & Egel-Mitani, 1974; see figure 1, g) if they are inoculated in rich media (with nitrogen) before they undergo meiosis so that for their maintenance it is necessary to keep them in rich medium. The diploid *S. pombe* cells are about 11-14 µm long after cell separation and 20-25 µ m before division. When the diploids are transferred to poor media they undergo meiosis and sporulate (figure 1, h).

As mentioned before, in addition to the heterothallic $h^+$ and $h^-$ strains, there are homothallic $h^{90}$ strains that are self-fertile due to a mating-type switching mechanism that allows them to switch between $h^+$ and $h^-$ every two generations. Thus, a unique cell is able to originate a colony of $h^+$ and $h^-$ cells that can conjugate to each other when nutrients are limited. In natural media, where the nutrients are scarce, it is also frequent to find pseudohyphal cells. This is interpreted as an adaptation to the unfavorable conditions. Recently, *S .pombe* has been described to be able to grow in the form of hypha under certain laboratory conditions on solid media containing an abundant carbon source, the basic nutrients for the growth, and limited nitrogen content (Amoah-Buahin *et al.*, 2005).

## 3. Cytoskeleton, cell wall and morphogenesis in *S. pombe*

Fission yeast is a unicellular, rod-shaped organism with a rigid cell wall. Its regular shape and genetic amenability have made it a useful organism for studying cell morphology. The cylindrical cells of *S. pombe* grow in a polarized and asymmetric way by extension of the ends and not the middle, and they divide by medial fission to generate two daughter cells of equal size. At the beginning of the cell cycle, growth is restricted to the *old end*, which existed in the previous cycle (figure 2). Later in the cell cycle, the *new end* formed from the septum starts to grow at a point in the cycle that is termed NETO (new end take-off) and the cells become bipolar (Mitchison & Nurse, 1985, Hayles & Nurse, 2001, La Carbona *et al.*, 2006). NETO is regulated by two control systems. First, cells must attain a critical length of 9.0-9.5µm and second, they must have completed some event in early $G_2$ (Mitchison & Nurse, 1985, Martin & Chang, 2005). Later, cells grow in a bipolar form, although the growth is faster at the old pole. The bipolar growth of the cells continues until they reach a critical cellular size, in which the growth ceases and the M (mitosis) phase of the cell cycle begins. This process culminates with the formation of a septum, consisting of cell wall material, between both daughter cells. Finally, cell separation occurs due to degradation of the septum, giving rise to two independent daughter cells (Hayles & Nurse, 2001, Martin & Chang, 2005, La Carbona *et al.*, 2006).

### 3.1. The actin cytoskeleton

Actin is encoded by a single gene ($act1^+$) and exists in two states: the globular actin (G-actin), which is the minor form, and the polymerized or filamentous actin (F-actin). Actin patches, actin cables and the contractile ring are comprised of F-actin. Different

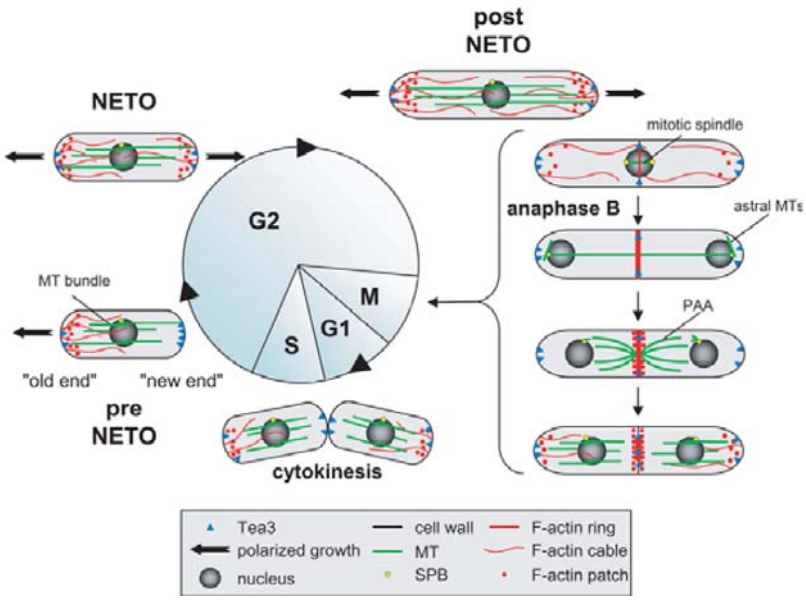



**Figure 2. Cytoskeletal rearrangements during the fission yeast cell cycle**. See the text for details. MT, microtubule, SPB, spindle pole body, NETO, new end takes off, PAA, post anaphase arrangement.

proteins associate with actin at these structures and regulate their stability (La Carbona *et al.*, 2006). Actin is an essential determinant for the polarized growth and morphogenesis in *S. pombe*; it localizes at the polarized growth zones and the division zone, and changes its distribution throughout the cellular cycle (figure 2). Actin patches concentrate at the growing cell pole(s), during cytokinesis they are observed at the cell equator and after septation actin patches concentrate at the old end which is the only tip to grow (La Carbona *et al.*, 2006).

Actin patches contain Arp2p (actin-related protein 2) and Arp3/Act2p, which belong to the Arp2/3 complex, the profilin Cdc3p and the coronin Crn1p. Their localization requires the Cdc8p tropomyosin. Actin patches are primary sites of Arp3-dependent actin nucleation and their maintenance, as well as that of actin cables, depends on a continuous actin polymerization (McCollum *et al.*, 1996, Morrell *et al.*, 1999, La Carbona *et al.*, 2006). Although actin patches are usually considered markers of the cellular polarity, there are some evidences suggesting that the correlation between the concentration of actin patches and the sites of cellular growth is not always true, since cells with delocalized actin patches are able to grow in a polar form (Morrell *et al.*, 1999, Feierbach & Chang, 2001). In agreement with this notion, it has been observed that the actin patches move towards the cellular interior from the cell tips or the septum, rather than concentrate in these zones (Feierbach & Chang, 2001, La Carbona *et al.*, 2006). Additionally, actin patches seem to mediate the internalization of endocytic vesicles. In fact, there is a correlation between the ratio of endocytosis at the cell ends (2:1, old end/new end) and the ratio of actin patches (Gachet & Hyams, 2005, Martin & Chang, 2005, Sirotkin *et al.*, 2005, La Carbona *et al.*, 2006).

Actin cables are bundles of filaments in whose assembly the formin For3p and the tropomyosin Cdc8p participate (Feierbach & Chang, 2001, Nakano *et al.*, 2002). Actin cables extend along the long axis in interphase, with their barbed end (the preferred end for polymerization) facing the nearest cell tip. They emanate from the cell poles and contribute to the polarized cellular growth, probably promoting and facilitating the localization of factors involved in polarity. At mitosis, cables are re-oriented such that the barbed end faces the mid-region of the cell, allowing the transport of material required for the formation of the contractile ring and septum towards the division site (Motegi *et al.*, 2001, Win *et al.*, 2001, La Carbona *et al.*, 2006, Mulvihill *et al.*, 2006, Fischer *et al.*, 2008). Then, they accumulate in the middle of the cell and arrange in order to form the actin ring. The contractile actin ring is a dynamic structure assembled in mitosis. This ring is associated with several proteins, such as the Arp2/3 complex, the proteins Cdc3p (profilin), Cdc12p (formin), Myo2p and Cdc4p (myosin II heavy and light chains, respectively; La Carbona *et al.*, 2006). Actin cables are important for

polarized growth, although there is probably another cellular polarization mechanism independent of the actin cables, since cells devoid of the formin For3p, and therefore of actin cables, exhibit growth problems but they do not show round morphology, as it would be expected in case of a complete absence of polarization (Feierbach & Chang, 2001, Nakano *et al.*, 2002).

### 3.2. Myosins

Myosins are motor proteins that associate with actin filaments and are formed by a complex of three proteins: a heavy chain, an essential light chain and a regulatory light chain. *S. pombe* possesses five myosins (Mulvihill & Hyams, 2003a, Gachet, 2004), two type-II myosins (Myo2p and Myo3/Myp2p), two type-V myosins (Myo51p and Myo52p) and a single type-I myosin (Myo1p). Myo2p, Myo3p and Myo51p are components of the contractile actomyosin ring (CAR) while Myo1p and Myo52p are involved in the deposition of the septum. The accumulation of all five myosins at the division plane in M phase depends on the septation initiation network (SIN), a signal transduction pathway that couples cytokinesis and exit from mitosis (Mulvihill & Hyams, 2003a).

In *S. pombe,* Myo1p class-I myosin is required for proper organization of the sterol-rich domains (Fischer *et al.*, 2008).

Myo2p and Myo3p are located exclusively at the equatorial zone of the cell and have no role other than cytokinesis. Myo2p is the only essential myosin in *S. pombe*, being necessary for cell division. Myo3p was initially suggested to have a role in cytokinesis only under certain stress condition, such as a high KCl concentration in the medium or a low temperature (Bezanilla *et al.*, 1997, Motegi *et al.*, 1997). Nevertheless, it was later described that Myo3p exerts a role in the division process, stabilizing the actomyosin ring during its contraction (Mulvihill & Hyams, 2003b). Additionally, the fact that the *myo2-E1myo3Δ* double mutant shows a higher defect in cell division than the corresponding single mutants suggests that both type-II myosins must collaborate in the cytokinesis during normal vegetative growth (Bezanilla *et al.*, 2000, Motegi *et al.*, 2000). The essential and regulatory light chains for the type-II myosins are coded by *cdc4*$^{+}$ and *rlc1*$^{+}$, respectively, and are also required for proper cytokinesis (McCollum *et al.*, 1995, Motegi *et al.*, 2000, Naqvi *et al.*, 2000).

Type-V myosins are responsible for the transport of vesicles and organelles within cells (Vale, 2003, Mulvihill *et al.*, 2006). The myosin tail acts as both, localization and cargo-binding domain (Mulvihill *et al.*, 2006). Myo51p relocates from the cell poles, where it plays a role in the maintenance of growth polarity at interphase, to the cell equator during division. In the absence of the type-V myosin Myo52p, CAR contraction is slower and discontinuous, and Myo51p accumulates in cytoplasmic vesicles. These findings demonstrate the interdependence of CAR contraction and septum deposition and show that Myo52p links the two processes (Motegi *et al.*, 2001, Mulvihill & Hyams, 2003a). Myo52p is required to deliver at least one of the glucan synthase enzymes to the inwardly-constricting CAR and, in consequence, to the incipient site of septal growth. The β(1,3)glucan synthase Bgs1p localizes to the cell tips and the division site and is required for the maintenance of polarized cell growth and septal synthesis. Bgs1p fails to localize normally in cells lacking Myo52p, suggesting that Myo52-dependent transport processes are required for Bgs1p to efficiently localize to regions of cell wall deposition (Le Goff *et al.*, 1999, Win *et al.*, 2001, Cortes, 2002, Mulvihill *et al.*, 2006).

### 3.3. The microtubule cytoskeleton

Microtubules (MTs) are hollow, cylindrical polymers that are found in all eukaryotic cells and are formed by the non-covalent association of tubulin molecules (Sawin & Tran, 2006). In the fission yeast, the microtubule cytoskeleton has a key role in cell morphogenesis by modulating spatial and temporal aspects of actin organization during the cell cycle and by targeting polarity factors involved in cell growth to the cortex. In this way, MTs contribute to establish an axis of polarity along the cell (Martin & Chang, 2005, La Carbona *et al.*, 2006, Sawin & Tran, 2006, Fischer *et al.*, 2008).

In interphase, MTs are organized in three to five anti-parallel bundles that arise from the central perinuclear regions and extend dynamically towards

the cell tips (Sato & Toda, 2004, Martin & Chang, 2005). The growth and shrinkage of these MTs are spatially regulated: MTs shrink back after contacting the cell tip for 1–2 minutes. Mutants defective in MT nucleation and dynamics exhibit aberrant cell shapes such as branched, rounded, or curved cells, suggesting that this regulated behavior of microtubules is critical for their function in cell polarity (Martin & Chang, 2005, Fischer *et al.*, 2008). During vegetative growth, three different types of MT organization, nucleated by three different types of microtubule organizing centres (MTOCs; figure 3) are present. During interphase, MTs can be nucleated not only from the spindle pole body (SPB) but also from additional sites on the nuclear surface, on MTs themselves, and in the cytoplasm. These non-SPB sites are collectively known as interphase MTOCs (iMTOCs; Sawin & Tran, 2006). As fission yeast undergoes "closed mitosis" in which the nuclear envelope does not break down, two SPBs nucleate bipolar spindles inside the nucleus during mitosis. In late anaphase, when the anaphase spindle is about to reach the cell end, another MT nucleation site called eMTOC (equatorial MTOC) emerges at the central region of the cell, thereby determining the division site of two daughter cells in the subsequent cytokinesis. MT nucleation sites such as SPBs and eMTOC contain γ-TuC (the γ-tubulin complex) from which MTs are nucleated. eMTOC forms a postanaphase array of MTs, and is required to maintain the contractile ring at the cell center prior to cytokinesis (Pardo & Nurse, 2003, Sato & Toda, 2004).

Because of the nature of their assembly from α, β-tubulin dimers, MTs are polar structures, with two distinct ends (see figure 3). These have been designated *plus* and *minus* ends, based on polymerization kinetics *in vitro* (Sawin & Tran, 2006). *In vivo*, MTs are rather stable at the minus end and are dynamic mainly at the plus end, which exhibits alternating rounds of growth and shrinkage (Sawin & Tran, 2006, Fischer *et al.*, 2008). In *S. pombe*, interphase MTs have a function in signaling polarity information to the cell ends. MT plus ends normally keep elongating until they reach the cell ends, and then shrink. This intrinsic characteristic of MTs allows them to transport and deliver the cell-end marker protein Tea1 to the cell ends (Fischer *et al.*, 2008).

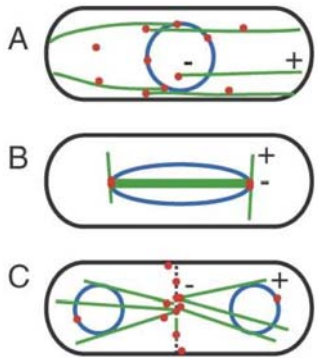



**Figure 3**. **Microtubule organization in *S. pombe***. A highly schematic illustration of microtubule (MT) distribution (green) in relation to microtubule organizing centres (MTOCs; red) and the nuclear envelope (blue). MT minus-ends ('−') are generally found towards the cell centre and MT plus-ends ('+') towards cell tips. During mitosis (B), intranuclear MTs form the mitotic spindle and astral MTs are nucleated from the SPBs. At the end of mitosis, during cell division (C), the equatoral MTOCs forms at the division site, to nucleate post-anaphase array MTs.

### 3.4. Biosynthesis of the cell wall in *S. pombe*

The cell wall is the exoskeleton of fungal cells, which confers osmotic protection and is essential for the cell integrity and survival. This structure is involved in active cellular processes such as conjugation, and is an essential morphogenetic element that is responsible for the final form of the organisms that possess it. Cell growth in *S. pombe* is a complex process necessarily related to cell wall biosynthesis (Ishiguro, 1998, Durán & Pérez, 2004). Cell wall is a dynamic structure that allows the morphological changes that occur during the life cycle, such as monopolar or bipolar growth, cytokinesis by medial fission, zygote formation, and spore development (Durán & Pérez, 2004). During these morphological changes, cell wall synthesis and degradation processes take place and these processes are regulated and coordinated with other cellular events and the cell cycle control machinery.

Cell wall is necessary to maintain the typical morphology of the *S. pombe* cells and for cell viability, as the protoplasts obtained by enzymatic digestion of the cell wall polymers are rounded up and quickly lose cell viability if not kept in an osmotically-stabilized medium. Thus, in addition to the interest of studying the synthesis and regulation of the cell wall as a morphogenetic model, we must not forget that the cell wall is an essential structure for fungal pathogens and is absent in higher eukaryotic cells. Deep study of

this structure is important to identify new targets for the development of antifungal compounds. In fact, one of the most promising antifungal is caspofungin acetate (CANCIDAS™), an inhibitor of the β-glucan biosynthesis which is currently being used for the treatment of invasive *aspergillosis* and other fungal infections (Wagner *et al.*, 2006).

### 3.4.1. Cell Wall composition and structure

The major *S. pombe* cell wall structural components are two glucose homopolymers; β(1,3)glucan, which constitutes about 50-54% of the total cell wall polysaccharides, and α(1,3)glucan, which constitutes about 28-32% of the total polysaccharides. Both these major glucans have high crystallinity and form the framework of the cell wall. Galactomannan is a non-structural polymer that represents 9-14% of the cell wall polysaccharides and is linked to proteins forming glycoproteins. Finally, β(1,6)glucan constitute about 2% of the total polysaccharides (Horisberger *et al.*, 1978, Humbel *et al.*, 2001, Durán & Pérez, 2004). In *S. pombe,* no chitin has been detected in the cell wall of vegetative cells but it appears on the cell wall of ascospores (Durán & Pérez, 2004). Electron microscopy of *S. pombe* cell wall shows a three-layered structure: two electron-dense layers separated by a non-dense layer which is mainly formed by β(1,3) glucan and a(1,3)glucan. Galactomannan is localized to the outer and inner layers (Humbel *et al.*, 2001, Durán & Pérez, 2004). The ultrastructure of the division septum is also very organized and has an electron-transparent inner layer (known as the primary septum) and two electron-dense layers on both sides of the primary septum with a composition similar to that of the lateral cell wall (known as the secondary septum. Humbel *et al.*, 2001. See figures 4 and 5).

**–β-glucan** is made of monomers of D-glucose united by β(1,3) or β(1,6) links. There are three different types of β-glucans: β(1,6)branched β(1,3)glucan, β (1,6)glucan and linear β(1,3)glucan.

**Linear β(1,3)glucan** is composed of linear chains of glucose without branches. It is exclusively present in the primary septum of dividing cells and its synthesis plays a relevant role in cell division (Cortes *et al.*, 2007. See figure 4).

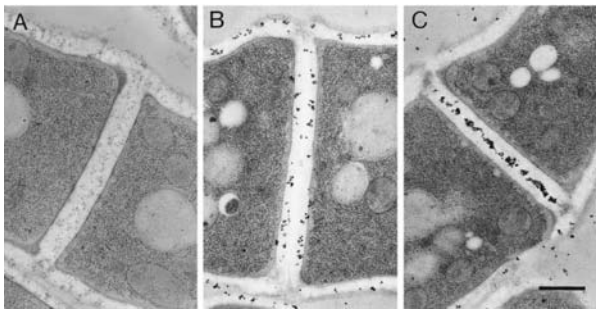



**Figure 4**. **Distribution of different types of glucan in the cell wall of *S. pombe*.** Colloidal-gold labeled antibodies were used for detection of different cell wall components using electron microscopy. Sections were labeled for β(1,6)-branched β(1,3) glucan (A), β(1,6)glucan (B) and linear β(1,3)glucan (C). Images taken from Humbel *et al*., 2001.

**β(1,6)-branched β(1,3)glucan** is the most abundant of all the polysaccharides and is build-up of glucose chains linked by β(1,3) bonds and branched through β(1,6) bonds, forming filamentous structures all over the cell wall non-dense layer. Protoplast regeneration studies have shown that β(1-3)glucan is the first polymer to be synthesized, forming a microfibrillar network composed of long parallel microfibrils resulting in ribbon-shaped bundles that are interwoven, and around it α-glucan and galactomannan are assembled. It is located across the cell wall, in the primary as well as in the secondary septa (Osumi, 1998, Osumi *et al.*, 1998, Humbel *et al.*, 2001, Durán & Pérez, 2004).

**β(1,6)glucan** polymer is made up of glucose chains linked through β(1,6) bonds and is highly branched, with up to 43% of β(1,3) links (Konomi *et al.*, 2003, Sugawara *et al.*, 2004). β(1,6)glucan is located in the outer part of the less dense layer, underneath the α-galactomannan layer of the lateral wall, and in the secondary septum (Humbel *et al.*, 2001. See figure 4). This location is in agreement with the hypothesis that β(1,6)glucan connects the mannoproteins to the cell wall. β(1,6)glucan was also found associated with the Golgi apparatus, in small vesicles and beneath the plasma membrane, indicating that β(1,6) glucan is synthesized in the endoplasmic reticulum–Golgi system and exported to the cell surface (Humbel *et al.*, 2001).

**–α-glucan** is present in the cell wall of *S. pombe* and other dimorphic and filamentous fungi (Bush *et al.*, 1974, Bobbitt *et al.*, 1977), but is curi-

ously absent from the *S. cerevisiae* cell wall. In *S. pombe,* α-glucan chains are formed by D-glucose united through α(1,3) links with 7% of α(1,4) bonds (Manners & Meyer, 1977, Grun *et al.*, 2005). The α (1,3)glucan consists of a short-straight chain structure of approximately 200 glucose residues (Konomi *et al.*, 2003). It has been observed that the α-glucan is essential for maintaining cell morphology (Alfa *et al.*, 1993, Cortes *et al.*, 2007). During the process of protoplast production, using different enzyme cocktails to digest the cell wall, Alfa *et al*. (Alfa *et al.*, 1993) showed that treatment with β-glucanase weakened the wall, but cells maintained the shape and it was only when they added α-glucanase that cells lost their polarity and became rounded.

The TEM images of the cells immunostained with anti-α(1,3)glucan antibody showed colloidal gold particles all over the cell wall. Several of these particles were observed on the surface and interior of the cell wall, but most were located along the cell membrane and seemed to enclose the cytoplasm. Colloidal gold particles are visible inside and outside the lipid bilayer of the membrane. It is known that α(1,3)glucan synthase localizes on the cell membrane and polymerizes glucoses inside the membrane before transporting them to the outside (Sugawara *et al.*, 2003).

**–Galactomannan and Mannoproteins** consists of α (1,6)-linked backbone with (1,2)-linked mannose side chains having nonreducing terminal galactose units which are α-linked (Horisberger *et al.*, 1978). This polymer is covalently attached to proteins by N- and O- glycoside links. These proteins are secreted into the periplasmic space and are located onto the outside of the cell wall and in the region nearest the plasma membrane. Galactomannan is present in the septum but only near the plasmalemma. A small amount of galactomannan is present in the cytoplasm of *S. pombe*, and thus it would appear that galactomannan is synthesized in or near the plasmalemma (Horisberger *et al.*, 1978, Andreishcheva *et al.*, 2004). The outer layer of glycoproteins is essential, among other processes, for the recognition of certain pathogenic fungi by the immune system and for the recognition of cells belonging to the opposite mating type during the sexual response (Hoyer, 2001, Verstrepen & Klis, 2006).

**–Chitin** is built-up of linear chains of N-acetyl-D-glucosamine united by β(1,4) links. Although some authors have identified residues of glucosamine in the cell wall of *S. pombe* (Sietsma & Wessels, 1990), it has not been possible to detect the presence of chitin through studies of X-ray diffraction or by staining with wheat germ agglutinin (WGA, which binds to chitin). The only stage at which chitin is present seems to be in the ascospores and could correspond to chitosan, which is deacetylated chitin. Chs1p has been identified as the chitin synthase responsible for the synthesis of chitin during sporulation (Arellano *et al.*, 2000).

Microscopy studies have shown that protoplasts regenerate with polarity, through the formation of a network of β-glucan fibrils appearing at a pole and extend outwards until they cover the surface of the protoplast and regenerate the cylindrical shape (Osumi, 1998, Durán & Pérez, 2004). In mutants with defects in α-glucan synthesis, the β-glucan fibrils do not develope into bundles, so the α-glucan could intervene in the β-glucan compaction. It seems that α-glucan is involved in the initial step of glucan bundle formation, framing the cell wall and strengthen it, and it acts in conjunction with β(1,3)glucan to maintain the cell shape (Konomi *et al.*, 2003). The spaces between the glucan fibers are filled with galactomannan (Osumi, 1998). Using β(1,3)glucanase and an inhibitor of β(1,3)glucan synthesis, aculeacin A, it has been shown that β(1,3)glucan was the main component of the fibrillar network structure in *S. pombe* (Osumi *et al.*, 1989, Osumi *et al.*, 1995, Konomi *et al.*, 2003). During spore development, the synthesis of β-glucan also precedes that of α-glucan and chitin (Martín *et al.*, 2000, Garcia *et al.*, 2006).

With regard to β-(1,6)glucan, this polymer could connect the outer layer of glycoproteins with the inner layer of polysaccharides of the cell wall, although the evidences supporting this hypothesis are not as clear as in *S. cerevisiae* (Roh *et al.*, 2002, Sugawara *et al.*, 2004).

Figure 5 shows the structure of the cell wall in *S. pombe*.

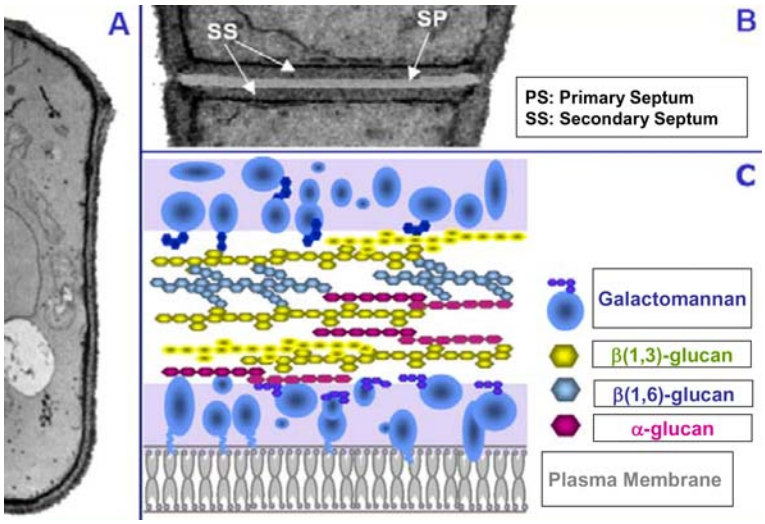



**Figure 5: Structure of the *S. pombe* cell wall.** The side wall consists of three layers (A and C). The layer less dense to electrons is composed of α and β glucans. The external layers are formed by galactomannan. Septum (B) presents an inner layer, mainly composed of linear β(1,3)-glucan (primary septum, PS), flanked by the secondary septum (SS) on both sides.

### 3.4.2. β(1,3)glucan biosynthesis in *S. pombe*

The biosynthesis of β-glucan is catalyzed by an enzyme-complex called β(1,3)glucan synthase (βGS) [UDP-glucose: (1-3)-β-D-glucan-3-β-D-glucosiltranferasa, EC 2.4.1.34]. This enzyme is linked to the plasma membrane and uses UDP-glucose as the substrate, forming linear chains of approximately 60-700 units of glucose. An *in vitro* system for the measurement of the synthesis of β-glucan has been developed. This system needs ATP and is activated by GTP. By using this system it was discovered that the βGS activity consists of at least two components: a catalytic and a regulatory fraction (Cabib *et al.*, 1998, Douglas, 2001).

In *S. pombe*, four genes have been identified that encode potential catalytic subunits of the β(1,3) glucan synthase complex: *bgs1*$^+$, *bgs2*$^+$, *bgs3*$^+$ and *bgs4*$^+$ (*bgs* stands for β-glucan synthase). *bgs1*$^+$, *bgs3*$^+$ and *bgs4*$^+$ are essential for viability and *bgs2*$^+$ is essential for sporulation (Ishiguro *et al.*, 1997, Liu *et al.*, 2000a, Martín *et al.*, 2000, Martín *et al.*, 2003, Cortes *et al.*, 2005, Cortes *et al.*, 2007). Bgs proteins are transmembrane proteins with high molecular weight (about 200k Da) and show similarity to Fks1p and Fks2p, the β-glucan synthases described in *S. cerevisiae* (Mazur, 1995). Some of these genes (*bgs3*$^+$ and *bgs4*$^+$) were identified studying mutants resistant or sensitive to antifungal compounds that inhibit cell wall synthesis (Martín *et al.*, 2003, Cortes *et al.*, 2005).

***–bgs1*$^+$*/cps1*$^+$:** The *S. pombe cps1-12* (*cps* stands for chlorpropham supersensitive) mutant strain was originally isolated as hypersensitive to the spindle poison isopropyl N-3-chlorophenyl carbamat (chlorpropham) and to the inhibitor of calmodulin trifluoperazine (Ishiguro & Uhara, 1992, Ishiguro *et al.*, 1997). Bgs1/Cps1p is assumed to be a component of the *S. pombe* β(1,3)glucan synthase (Liu *et al.*, 2002, Cortes *et al.*, 2007). The *cps1-12* mutation confers hypersensitivity to the immunosuppressant cyclosporin A (CsA), a potent inhibitor of the $Ca^{2+}$/calmodulin-dependent protein phosphatase 2B calcineurin, and to papulacandin B, a drug that specifically inhibits β(1,3)glucan synthesis both *in vivo* and *in vitro*. The *cps1-12* mutant displays several phenotypes typical of cell wall synthesis defects, namely, increased sensitivity to enzymatic cell wall degradation and a thermosensitivity with lysis that can be prevented with an osmotic stabilizer. Also, the *cps1-12* mutant shows both altered β(1,3)glucan synthase activity and altered cell wall composition (Ishiguro *et al.*, 1997). Consistent with this, Cps1p is detected at the constricting actomyosin ring and the Cps1p ring contracts in concert with the centripetal deposition of β(1,3)glucan (Liu *et al.*, 2002). Levels of Cps1p do not fluctuate appreciably along the cell cycle. Thus, protein levels do not appear to determine Cps1p function. Rather, its function must be regulated by its intracellular localization and the activation of its enzymatic activity (Liu *et al.*, 2002). *bgs1Δ* deletion is ultimately lethal. Different experiments suggested that Bgs1/Cps1p might be responsible and essential for the synthesis of a specific lineal β(1,3)glucan that constitutes the fission yeast primary septum (Humbel *et al.*, 2001, Cortes, 2002). Linear β(1,3)glucan is the polysaccha-

ride that specifically interacts with the fluorochrome Calcofluor white so the septa in the *bgs1Δ* mutants do not stain with this dye (Cortes *et al.*, 2007). In the absence of Bgs1p, remedial septa lacking linear β(1,3) glucan are synthesized but they cannot be degraded properly and the cells remain chained (Cortes *et al.*, 2007). Thus, Bgs1p plays an important role in cytokinesis.

Bgs1/Cps1p has also been implicated in a cytokinesis checkpoint. The *cps1-191* mutant arrests with two interphase G2 nuclei and a stable actomyosin ring at the restrictive temperature (Liu *et al.*, 1999, Liu *et al.*, 2000b). The cytokinesis checkpoint might also prevent cell elongation in arrested *cps1-191* cells. However, the defect in G2/M transition in these cells is not due to insufficient cell mass increase, since elongated *cps1-191* cells shifted to the restrictive temperature still arrest with only two nuclei. It is likely that some aspect of completion of cytokinesis is checked by this cytokinesis monitoring system, which allows entry into the subsequent M phase only upon successful completion of cytokinesis. It therefore seemed possible that the continued presence of the actomyosin ring in interphase-arrested *cps1-191* cells prevented entry into a subsequent M phase. The checkpoint is activated in response to the persistence of some F-actin dependent structure that is normally disassembled upon completion of cytokinesis. Furthermore, the elements that control the G2 delay in response to damaged DNA and unreplicated DNA are not important for the G2 delay observed in the *cps1-191* mutants.

The nature of the signal that activates the cytokinesis checkpoint system is unclear. However, two possibilities are proposed based on data presented in (Liu *et al.*, 1999, Liu *et al.*, 2000b). Given that the *cps1-191* mutant arrests with a stable actomyosin ring and two G2 nuclei, one possibility is that the persistence of an actomyosin ring or other F-actin-dependent structures might prevent entry into a subsequent round of mitosis. Consistent with this, it has been found that a brief treatment of arrested *cps1-191* cells with the actin polymerization inhibitor Lat A allows at least 40% of cells to undergo a second round of mitosis. Alternatively, Cps1p itself might activate the cytokinesis monitoring system, since *cps1*-null mutants proceed through multiple mitotic cycles and accumulate up to 32 nuclei, whereas the *cps1-191* mutant arrests with two nuclei (Liu *et al.*, 1999, Liu *et al.*, 2000b). In this model, the product of the *cps1-191* allele is presumed only to be defective in the enzymatic synthesis of β(1,3)glucan, since it is capable of transmitting the signal resulting from the lack of septum assembly. By contrast, in the absence of Cps1p both enzymatic synthesis and perception of failed cytokinesis are affected (Liu *et al.*, 2000b).

Respect to its localization, Bgs1/Cps1p is an integral membrane protein that shows a localized pattern in all stages of vegetative growth (Cortes, 2002, Cortes *et al.*, 2007). It localizes to growing poles, the sites of cell wall synthesis during sexual differentiation (Cortes, 2002, Cortes *et al.*, 2007), and to the cell division site in a medial ring structure overlying the actomyosin ring, late in anaphase (Cortes, 2002, Liu *et al.*, 2002, Mulvihill *et al.*, 2006). F-actin is necessary for the localization, but not the maintenance, of Bgs1p at the cell division ring (Cortes, 2002, Liu *et al.*, 2002). Assembly of Bgs1/Cps1p into the cell division ring, at the appropriate time in the cell cycle, is also dependent on the septation-inducing network (SIN) proteins that regulate division septum formation after assembly of the actomyosin ring (see below). Cdc15p, an SH3 domain-containing component of the actomyosin ring, is essential for targeting Bgs1/Cps1p to the division site after the assembly of the actomyosin ring, which serves as a spatial landmark for the incorporation of Cps1p in a medial ring structure (Cortes, 2002, Liu *et al.*, 2002). Fluorescence-recovery after photobleaching experiments (FRAP) revealed that Bgs1/Cps1p does not diffuse appreciably within the plasma membrane, and that it must be retained at the division site by a novel F-actin- and microtubule-independent mechanism (Liu *et al.*, 2002). The secretion machinery is also essential for Bgs1/Cps1p localization (Liu *et al.*, 2002, Mulvihill *et al.*, 2006). Finally, Bgs1/Cps1p localization is very unstable and extremely sensitive to cell wall stress (Cortes, 2002). Consistent with its role in cell wall synthesis, Bgs1p co-localizes with Rho1p GTPase, the glucan synthase regulatory subunit (Arellano *et al.*, 1997), in the growing sites during the mitotic cycle (Cortes, 2002).

When *bgs1Δ* cells are kept alive in the presence of sorbitol, cells produce apical branches that grow in

opposite directions; this results show that Bgs1/Cps1p plays an important role in growth polarity and morphogenesis (Cortes *et al.*, 2007). Additionally, it has been shown that the Myo52-dependent transport of Bgs1p is an important element in the maintenance of growth polarity in the fission yeast (Mulvihill *et al.*, 2006).

***–bgs2***$^{+}$ gene was identified in the sequencing project of the *S. pombe* genome as a gene coding for a protein that shares similarity with Bgs1p from *S. pombe* and with Fks1p from *S. cerevisiae*. *bgs2*$^{+}$ is only expressed during sporulation and is essential for this process. *bgs2Δ* mutants show a reduction in the βGS activity and ascospores lacking Bgs2p are immature, are not released from the asca, and are not able to germinate. Electron microscopy showed that in the *bgs2Δ* mutant the spore wall is thinner than in the WT strain, and that the β-glucan layer is barely detected (Liu *et al.*, 2000a, Martín *et al.*, 2000) .

***–bgs3***$^{+}$ was identified as a gene that was able to suppress the phenotype of hypersensitivity to antifungals (Calcofluor and Echinocandin) exhibited by the *ehs2-1* (*e*chinocandin *h*iper*s*ensitive) mutant. Bgs3p is necessary to maintain the cell viability and polarity during vegetative growth. Cells in which the expression of *bgs3*$^{+}$ is turned off (by expressing this gene under the control of the thiamine-repressible promoter 81Xnmt) are rounded, and their cell walls are thicker than those of the control strain and exhibit an increased amount of α-glucan, probably to compensate the decrease in the amount of β-glucan (Martín *et al.*, 2003).

Similarly to Bgs1p, Bgs3p is detected at the septal region during cell division and at the cell poles during interphase (Martín *et al.*, 2003). Although Bgs3p function is unknown, its presence is required for survival (Martín *et al.*, 2003), and its expression is increased when there are defects in the synthesis of other glucan synthases (Bgs1p and Bgs4p), which suggests that Bgs3p might play a role in a compensatory mechanism that would work in response to cell wall damage (Cortes, 2006).

***–bgs4***$^{+}$ was identified as the gene altered in the *cwg1-1* mutant, which shows a reduction in βGS activity and lyses massively at 37ºC (Ribas *et al.*, 1991). Bgs4p is essential for spore germination. Microscopic observations showed that *bgs4Δ* spores are able to germinate but that they lyse before the first round of cell division. Bgs4p is also essential for maintaining cell integrity during both cytokinesis and polarized growth. Morphological analyses showed that *bgs4*$^{+}$ repression promoted cell lysis and the release of cytoplasmic material at the regions of cell wall synthesis, either the poles or the septum. However, the function of Bgs4p at the cell wall-septum boundary is more critical than that at the poles (Cortes *et al.*, 2005). Thus, Bgs4p has been suggested to be the main βGS involved in the synthesis of an essential β(1,3)glucan and also in a crucial repair mechanism against a possible excess of cell wall degradation during cell division (Cortes *et al.*, 2005).

Bgs4p localizes to the growing ends, the medial ring and septum, and at each stage of wall synthesis or remodeling that occurs during sexual differentiation. Bgs4p localization during septation depends on the medial ring but not on the septation initiation network. The actin cytoskeleton is necessary for Bgs4p delocalization and relocalization but not for its maintenance at the growing sites (Cortes *et al.*, 2005). Some of these requirements have also been reported for other Bgs/Fks homologues. F-actin is necessary for the localization, but not the maintenance, of Bgs1p at the cell division ring; it is not required for Bgs3p maintenance at the growing sites, and it is needed for Fks1p depolarization and repolarization (Delley & Hall, 1999, Liu *et al.*, 2002, Martín *et al.*, 2003).

### 3.4.3. Role of Rho-GTPases in cell wall synthesis

Approximately one percent of the human genome encodes proteins that either regulate or are regulated by direct interaction with members of the Rho family of small GTPases. Through a series of complex biochemical networks, these highly-conserved molecular switches control some of the most fundamental processes of cell biology common to all eukaryotes, including morphogenesis, polarity, movement, cell division, vesicular trafficking, cell cycle, and transcriptional dynamics (see Jaffe & Hall, 2005, Bustelo *et al.*, 2007 as reviews).

In yeast, Rho-type GTPases control cell integrity by coordinating cell wall synthesis and are essential for cell survival through the regulation of the actin cytoskeleton, microtubules, and secretion. Mutants in the Rho-family GTPases are defective in different aspects of these cellular processes; show aberrant morphologies and are defective in cell division and in maintenance of cell wall integrity (see Bustelo *et al.*, 2007, Park & Bi, 2007 as reviews).

*S. pombe* contains six GTPases of the Rho family; Rho1p, Rho2p, Rho3p, Rho4p, Rho5p and Cdc42p. They are involved in the maintenance of cell morphogenesis by regulating different aspects of cell polarity (Cdc42p), in the maintenance of cell integrity during cell growth and division (Rho1p, Rho2p and Rho5p), and in secretion (Rho3p and Rho4p). All of them affect the regulation of the actin cytoskeleton and are localized in the places of active growth (Miller & Johnson, 1994, Nakano *et al.*, 1997, Hirata *et al.*, 1998, Calonge *et al.*, 2000, Nakano *et al.*, 2003, Santos *et al.*, 2003, Nakano *et al.*, 2005, Santos *et al.*, 2005, Rincon *et al.*, 2006).

**–*rho1*$^+$:** Cell growth in *S. pombe,* as well as in *S. cerevisiae,* is necessarily related to cell wall synthesis and to the regulation of the actin cytoskeleton. The Rho1p GTPase is involved in both processes. In *S. cerevisiae*, Rho1p regulates the β-glucan synthesis directly by activating Fks1p and Fks2p, the catalytic subunits of the β-glucan synthase enzyme and indirectly by activating the MAP kinase pathway that controls cell integrity), which is composed by the protein kinase C-homologue Pkc1p, a module of MAP kinases, and the transcription factors Rlm1p and Swi4/6, (see Levin, 2005 as a review).

In *S. pombe*, Rho1p acts as a regulatory subunit for the β(1,3)glucan synthase activity. It activates the synthesis of β(1,3)glucan either directly or through the two protein kinase C-homologues, Pck1p and Pck2p (Arellano *et al.*, 1996, Arellano *et al.*, 1999b. See figure 6). *rho1*$^+$ is an essential gene. Cells lacking *rho1*$^+$ (by turning off the 41Xnmt thiamine-repressible promoter) lose their integrity and lyse, mostly during cell separation. Before cell lysis starts, actin depolymerizes and actin cables and patches disappear. The βGS activity also decreases drastically (Arellano *et al.*, 1997, Arellano *et al.*, 1999a).

**–*rho2*$^+$:** Rho2p interacts with the protein kinase C-homologues, Pck2p and Pck1p (figure 6) and, like Rho1p, is required for the maintenance of cell integrity. *rho2Δ* mutants show a rounded morphology and exhibit sensitivity to drugs that affect the cell wall synthesis (Hirata *et al.*, 1998). Rho2p is involved in the regulation of α-glucan synthesis through Pck2p (Calonge *et al.*, 2000). In this way, Rho2p and Rho1p regulate the synthesis of major cell wall polymers, α- and β-glucan, through Pck2p (Arellano *et al.*, 1996, Arellano *et al.*, 1999b, Perez & Calonge, 2002).

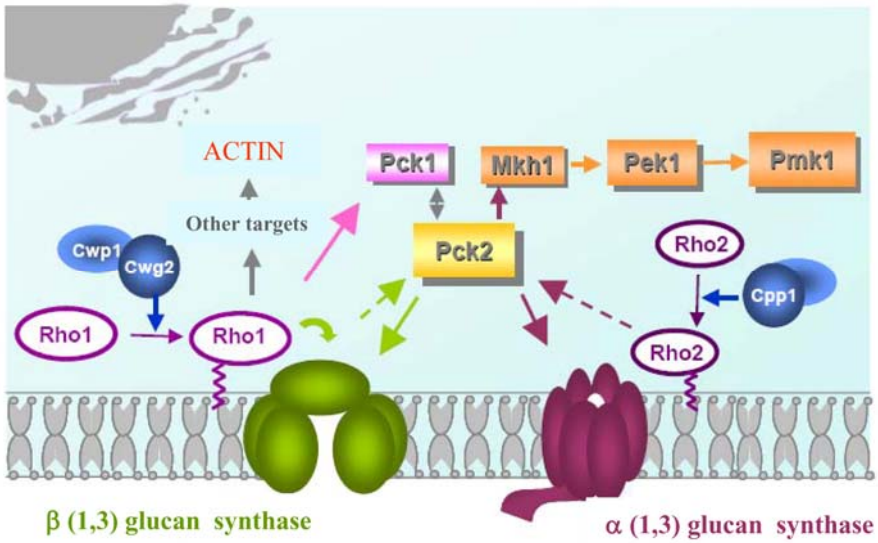



**Figure 6**. **Regulation of cell wall synthesis in *S. pombe***. The functional relationships between glucan synthases, GTPases, PKC-homologues and the cell integrity pathway are shown.

### 3.5. *CHS* genes, cell wall synthesis and morphogenesis in yeasts

The organism in which cell wall composition, regulation and biosynthesis is best-characterized is *S. cerevisiae* (see reviews Orlean, 1997, Valdivieso, 2004). In this organism, cell wall is composed of β-glucan, chitin and mannoproteins. Three chitin synthase (CS) activities are responsible for the synthesis of chitin: CSI, which is responsible for a repairing activity that counteracts a possible excessive action of chitinase during cell separation; CSII, which is the only CS responsible for the synthesis of the primary septum, and CSIII, which is responsible for chitin synthesis in the lateral wall, shmoo, and the wall of the spores. The absence of CSIII activity leads to a decrease in the amount of chitin, which confers resistance to Calcofluor, a 50% reduction in the efficiency of conjugation, and a defect in the maturation of spores (for review see Valdivieso, 2004). While for the CSI and II only the structural genes have been identified (*CHS1* and *CHS2*, respectively), for the CSIII activity several proteins that regulate Chs3p (the catalytic subunit) have been identified. The genes that participate in this activity are described below:

**–*CHS7*:** This gene encodes an integral membrane protein that is located in the endoplasmic reticulum. Chs7p acts as a specific chaperone for Chs3p, allowing its sorting from this organelle.

**–*CHS5* and *CHS6*:** These genes encode late Golgi (TGN)/early endosome-compartment proteins required for the transport and recycling of Chs3p (Santos *et al.*, 1997, Santos & Snyder, 1997, Sanchatjate & Schekman, 2006). It has recently been described that Chs5p is an adaptor protein that mediates the binding of Chs6p and its three paralogues (Bch1p, Bud7p and Bch2p; generically named ChAPs) to Chs3p and the Arf1p GTPase. It seems that Chs5p and the ChAps form a coat (the exomer) on a particular population of TGN vesicles, in which Chs5p serves as an anchor and the different ChAPs could be responsible for the cargo specificity. The phenotype of each mutant is specific, while the *chs5Δ* mutant shows the phenotypes of all of them. Neither the deletion of each ChAPs gene nor the quadruple mutants are lethal (Trautwein *et al.*, 2006, Wang *et al.*, 2006). Chs5p has a fibronectin domain type III (FN3) and a BRCT domain on its amino terminal end. This area of the protein is required for QSIII activity (Wang *et al.*, 2006). Furthermore, Chs5p but not the ChAPs is required for conjugation, being necessary for transporting Fus1p (a membrane protein required for cell fusion during conjugation) to the tip of the shmoos (Santos *et al.*, 1997, Santos & Snyder, 1997, Santos, 2003).

**–*CHS4*:** Chs4 proteins from *Saccharomyces cerevisiae* and from *Candida albicans* are chitin synthase regulators (Trilla *et al.*, 1997, Sudoh, 1999). 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 (DeMarini, 1997, Trilla *et al.*, 1997, Reyes *et al.*, 2007). Additionally, Chs4p participates in mating, since *chs4Δ* mutants shows a reduction in the formation of diploids more drastic than the *chs3Δ* mutants. This defect is prior to the cell fusion step of mating, unlike that of the *chs5Δ* mutants (Trilla *et al.*, 1997).

Chs4p belongs to a family of proteins called "solenoids" due to the three-dimensional structure that confers the presence of SEL1 domains. These domains are modules for protein-protein interactions that facilitate interaction with specific proteins. This family of proteins participates in different cellular processes, such as cell cycle, ubiquitination, transcription and protein delivery, and is present in all the biological scale except for *Archaeobacteria* (Mittl & Schneider-Brachert, 2006). Some members of this family of proteins also share the presence of a prenylation site. In the case of *S. cerevisiae*, a Chs4 protein lacking the prenylation site was found to be able to complement a *chs4Δ* mutant (DeMarini, 1997, Reyes *et al.*, 2007), although this domain seems to be required for a robust chitin synthase III activity (Grabinska *et al.*, 2007). In *S. cerevisiae*, the region of Chs4p containing the SEL1 domains is able to complement a *chs4Δ* mutant (DeMarini, 1997, Ono N, 2000, Reyes *et al.*, 2007). The region of the *Candida albicans* Chs4 protein that contains the SEL1 domains is able to complement the *S. cerevisiae chs4Δ* mutant (Sudoh, 1999).

Shc1p is a protein homologue to Chs4p necessary for chitin synthesis in the spores. Its ectopic expression during the vegetative growth is able to activate Chs3p, but not to localize it in the neck. Chs4p contains seven SEL1 domains, which seem to be required for the activation of the Chs3p enzymatic activity (Ono N, 2000) and for its proper location in the neck (DeMarini, 1997). Since Shc1p has only four SEL1 domains, it has been suggested that the three other domains would be responsible for the interaction between Chs4p and Bni4p.

ORF YDL203c, which was found after sequencing the *S. cerevisiae* genome, encodes a protein that shares 28% identity with Chs4p and contains seven SEL1 domains. In our laboratory we have found that this protein is located at the sites of polarized growth and that the single mutant and the triple *ydl203 chs4 shc1* mutants are viable.

**–*CHS*-like genes in *S. pombe*:** It has not been possible to establish the presence of chitin in the *S. pombe* vegetative cell wall (Horisberger & Rouvet-Vauthey, 1985, Sietsma & Wessels, 1990). However, a CS activity has been found in this fission yeast (Sietsma & Wessels, 1990, Arellano *et al.*, 2000). In the genome of this organism, there are two genes with similarity to CS genes from other fungi. *chs1*$^+$ shares 47%, 45% and 28% identity with the Sc*CHS2,* Sc*CHS1* and Sc*CHS3* genes, respectively, and codes for a protein responsible for the CS activity (Arellano *et al.*, 2000). Indeed, this enzyme turned out to be a *bona fide* CS enzyme required for proper maturation of the ascospores (Arellano *et al.*, 2000). *chs2*$^+$ is 32% and 29% identical to Sc*CHS2* and Sc*CHS1*, respectively, while it has no significant identity with the Sc*CHS3* gene. It is noteworthy that Chs2p lacks the QRRRW domain and several amino acids essential for CSII activity and, in agreement, it has no CS activity (Martin-Garcia *et al.*, 2003). This protein has been shown to be a linker between the plasma membrane and the myosin component of the contractile actomyosin ring and to stabilize this ring during cytokinesis (Martin-Garcia & Valdivieso, 2006). In parallel, a similar role was found for the *S. cerevisiae* Chs2 protein (VerPlank & Li, 2005), showing that both proteins share a function in stabilizing the ring during contraction.

In *S. cerevisiae*, Chs5p is a Golgi protein required for the transport of the CS Chs3p to the plasma membrane (Santos *et al.*, 1997, Santos & Snyder, 1997). It is also necessary for the transport of Fus1p to the tip of the shmoos during mating, so a *chs5Δ* mutant exhibits a mating defect that is independent of the role of Chs5p in chitin synthesis (Santos *et al.*, 1997, Santos & Snyder, 2003). *S. pombe* Cfr1p shares sequence similarity with Chs5p. Deletion of the *cfr1*$^+$ gene does not affect cell wall synthesis but results in a mating defect similar to that of the *S. cerevisiae chs5Δ* mutants (Cartagena-Lirola *et al.*, 2006).

## 4. Cytokinesis in *S. pombe*

Cytokinesis was the first cell cycle event to be described. It was described over one hundred years ago by embryologists studying eggs of amphibian and marine invertebrates. Cytokinesis is the cellular process by which eukaryotic cells divide after mitosis to form two daughter cells (Fishkind & Wang, 1995). Cytokinesis must be coordinated with the process of nuclear division or mitosis. It has been shown that the main components involved in the cytokinesis process are highly conserved from yeast to humans (see Balasubramanian *et al.*, 2004, Wolfe & Gould, 2005 as reviews). Fission yeast is a unicellular organism that exhibits the features of cytokinesis typical of other eukaryotic cells and, as it is amenable to genetic manipulation, is extensively used as a model organism to study this process.

Table 1 shows the main elements involved in the *S. pombe* cytokinesis, which will be mentioned in the paragraphs 4.1-4.6

### 4.1. Cell division site selection and positioning of the contractile ring

The proper positioning of the plane of cell division is critical for successful partitioning of daughter nuclei and other cytoplasmic organelles, as well as for normal cellular architecture and differentiation during development. The mechanism of determining the site of division seems to be a step in cytokinesis that is species-specific. In the fission yeast, the site of ring assembly correlates with the position of the interphase

**Table 1. Relevant *S. pombe* Genes involved in cytokinesis**

| Gene | Protein | Gene | Protein |
|---|---|---|---|
| **CAR components** | | **SIN pathway** | |
| *act1*$^+$ | Actin | *byr4*$^+$ | GAP |
| *adf1*$^+$ | Cofilin | *cdc7*$^+$ | Kinase |
| *ain1*$^+$ | Alfa-actinin | *cdc11*$^+$ | Scaffold |
| *arp2*$^+$ | Actin-related protein | *cdc14*$^+$ | Sid1p-binding protein |
| *arp3*$^+$ | Actin-related protein | *cdc16*$^+$ | GAP |
| *cdc3*$^+$ | Profilin | *mob1*$^+$ | Sid2p-binding protein |
| *cdc4*$^+$ | Myosin essential light chain | *plo1*$^+$ | POLO Kinase |
| *cdc8*$^+$ | Tropomyosin | *sid1*$^+$ | Kinase |
| *cdc12*$^+$ | Formin | *sid2*$^+$ | Kinase |
| *cdc15*$^+$ | PCH protein | *sid4*$^+$ | Scaffold |
| *chs2*$^+$ | Chitin synthase-like protein | *spg1*$^+$ | GTPase |
| *fim1*$^+$ | Fimbrin | **SIN-related components** | |
| *imp2*$^+$ | PCH protein | | |
| *myo2*$^+$ | Type-II myosin heavy chain | *dma1*$^+$ | Negative regulator |
| *myo3*$^+$ | Type-II myosin heavy chain | *etd1*$^+$ | Protein that binds Cdc15p |
| *myo51*$^+$ | Type-V myosin heavy chain | *flp1*$^+$ | Phosphatase |
| *myo52*$^+$ | Type-V myosin heavy chain | *lsk1*$^+$ | Kinase |
| *plo1*$^+$ | POLO Kinase | *par2*$^+$ | Phosphatase |
| *rlc1*$^+$ | Myosin regulatory light chain | *ppb1*$^+$ | Calcineurin |
| *rng2*$^+$ | IQGAP protein | *scw1*$^+$ | RNA-binding protein |
| *rng3*$^+$ | UCS-domain protein | *zfs1*$^+$ | Zinc finger protein |
| **CAR positioning** | | **Septum synthesis** | |
| *mid1*$^+$ | PH-Domain protein | *bgs1*$^+$ | β(1,3)glucan synthase |
| *plo1*$^+$ | POLO Kinase | *bgs4*$^+$ | β(1,3)glucan synthase |
| *pom1*$^+$ | Kinase | *mok1*$^+$ | a(1,3)glucan synthase |
| | | **Septum degradation** | |
| | | *agn1*$_+$ | α(1,3)glucanase |
| | | *eng1*$_+$ | Endo β(1,3)glucanase |
| | | *spn1*$^+$-*spn5*$^+$ | Septins |

nucleus, and is established in the G2 phase of the cell cycle. In *S. cerevisiae,* the division site is determined in the G1 phase by the position of the former site of budding. In animal cells, the future division site is determined during anaphase by the position of astral microtubules or the central spindle apparatus.

The fission yeast medial ring forms in early mitosis (Marks, 1985), coincident with spindle assembly, while in animal cells, the ring forms in late mitosis. While contractile ring formation is dependent on the mitotic spindle in animal cells, in fission yeast assembly and placement of the medial ring do not require a mitotic spindle (Chang & Nurse, 1996). In *S. pombe*, the position of the pre-mitotic nucleus defines the position of medial ring (Chang & Nurse, 1996). Two kinases, Pom1p and Polo kinase Plo1p, and the anillin-like protein Mid1p are involved in the correct positioning of the contractile actomyosin ring (CAR) with respect to the position of the nucleus in interphase (Ohkura *et al.*, 1995, Sohrmann *et al.*, 1996, Bähler & Pringle, 1998, Bahler *et al.*, 1998, Paoletti &

Chang, 2000). A clear role for Plo1p in proper spatial coupling of cell and nuclear division has been shown (Bahler *et al.*, 1998).

*mid1*$^{+}$ gene encodes a protein with a C-terminal pleckstrin homology (PH) domain, which are domains that occurs in a wide range of proteins involved in intracellular signaling or as constituents of the cytoskeleton (Sohrmann *et al.*, 1996). In interphase, Mid1p shuttles between the nucleus and the adjacent cortex and accumulates predominantly in the nuclear compartment. At the onset of mitosis, Mid1p is released from the nucleus in a Plo1p kinase-dependent manner and forms a broad cortical band that defines the site for recruitment of proteins involved in actomyosin ring assembly, thereby marking the site of cell division. This diffuse cortical band becomes a tight ring by the time of anaphase onset (Sohrmann *et al.*, 1996, Bahler *et al.*, 1998, Mulvihill *et al.*, 1999). Mid1p is responsible for recruiting and concentrating progenitors of the immature ring such as Myo2p, which has been described to interact directly with Mid1p, Cdc4p, Rng2p, Cdc15p, and Cdc12p at the cell cortex overlying the nucleus (see Wolfe & Gould, 2005 as a review).

Pom1p is a kinase that is localized in the equatorial zone of the cell and in the cell poles; this protein regulates positioning of the cell division site and polarized growth, possibly restricting the growth of microtubules (Bähler & Pringle, 1998). Although the position of the CAR is not determined directly by microtubules in fission yeast, these structures are involved in the position of the nucleus in interphase (Daga *et al.*, 2006) so that they are important for the positioning of the division site, and are required to keep the contractile ring in the cell middle (Pardo & Nurse, 2003).

### 4.2. Contractile ring assembly

The actomyosin ring is a key element in the cytokinesis process in both yeast and animal cells. Actomyosin-based rings underlying the plasma membrane are assembled between segregating copies of genetic material during eukaryotic cell division. The basic components of this structure are generally the same, but the order of assembly of these components in the ring varies depending on the organisms. CARs are dynamic, complex structures and their assembly and constriction in the plane orthogonal to mitotic spindles is tightly regulated in space and time.

The assembly of actomyosin structures can be triggered by two independent mechanisms: Mid1p release from the nucleus and the Septation Initiation Network (SIN) signaling. In wild type cells, Mid1p and SIN cooperate to orchestrate CAR assembly in mitosis (figure 7) and Plo1p coordinate Mid1p and the SIN pathway. These two regulators are partially redundant for the assembly of actomyosin structures, yet each has a specific role: Mid1p ensures correct CAR positioning, while the SIN ensures the formation of a functional CAR (Hachet & Simanis, 2008). However, neither Mid1p nor the SIN is considered to be essential for CAR assembly *per se* (Hachet & Simanis, 2008, Huang *et al.*, 2008).

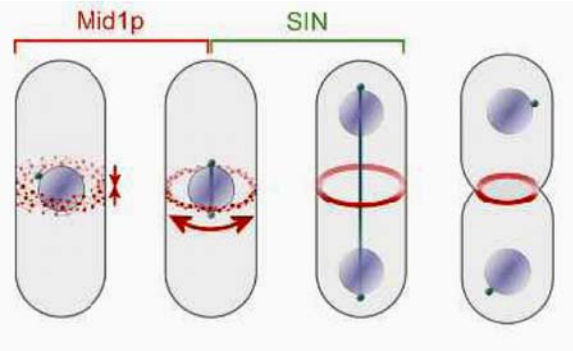



**Figure 7. Ring assembly during mitosis.** CAR assembly is initiated by the formation of a cortical network of ring components that undergoes lateral condensation, giving rise to a nonhomogeneous ring precursor. This structure eventually matures by recruitment of additional factors. The time of regulation of ring assembly by Mid1p or the SIN is indicataed. Taken from Hachet and Simanis, 2008.

Different studies have produced conflicting models for CAR assembly in fission yeast (figure 8); a *leading cable* mechanism, whereby cable emanate from a single nucleation site at the cell cortex (Chang *et al.*, 1997, Kamasaki *et al.*, 2007), and a *search/capture* mechanism that proceeds via the formation of a network of nodes that coalesce to form a CAR (Bahler *et al.*, 1998, Wu *et al.*, 2006). Depending on whether the Mid1p spatial cue is present, either mechanism can be used to form a functional CAR. In the absence of Mid1p, CAR assembly proceeds via an actomyosin filament, rather than a cortical network of CAR proteins. However, these CARs formed by what resembles the *leading cable* mechanism independently

of Mid1p contract more slowly. This mode of assembly is totally dependent on SIN signaling (Hachet & Simanis, 2008, Huang *et al.*, 2008). It is noteworthy that Clp1p/ Flp1p, which is important to assure stability of the CAR, is recruited to the CAR by Mid1p (Clifford *et al.*, 2008). SIN mutants fail to form a homogeneous CAR; hypophosphorylation and recruitment of the conserved PCH-family protein Cdc15p to the CAR is a critical step requiring SIN function (Hachet & Simanis, 2008).

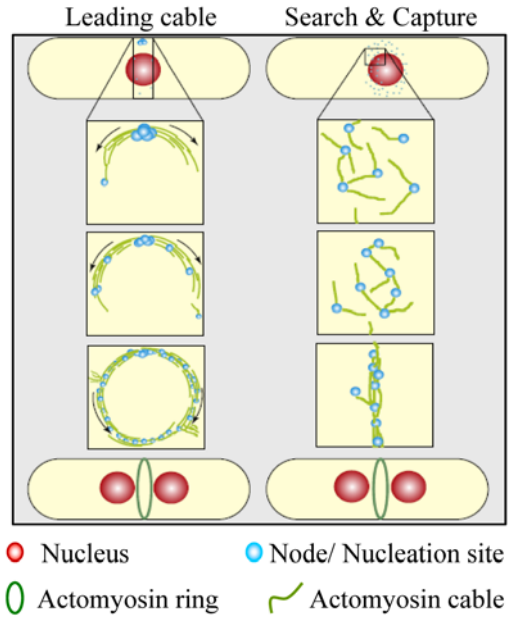



**Figure 8. Models of actomyosin ring assembly.** (A) The leading cable mode of ring assembly. (B) The search-and-capture mechanism. Taken from Mishra and Oliferenko, 2008.

Genic analyses have identified many components of the fission yeast medial ring required for assembly, placement and/or maintenance of the cell division ring (Nurse *et al.*, 1976, Chang *et al.*, 1996, Gould & Simanis, 1997, Balasubramanian *et al.*, 1998). These gene products include many conserved proteins involved in actin-organization (Field *et al.*, 1999). In addition to F-actin, the CAR contains numerous proteins, such as the formin Cdc12p (Chang *et al.*, 1997), the IQGAP-related protein Rng2p (Eng *et al.*, 1998), PSTPIP-related proteins Cdc15p and Imp2p (Fankhauser *et al.*, 1995, Demeter & Sazer, 1998), the tropomyosin homolog Cdc8p (Balasubramanian, 1992, Arai *et al.*, 1998), the profilin Cdc3p (Balasubramanian, 1994), the essential myosin light chain Cdc4p (McCollum *et al.*, 1995), Rng3p which is the factor involved in assembly and correct conformation of the myosin ring (Wong *et al.*, 2000), and type-II myosin heavy chain, Myo2p (Kitayama *et al.*, 1997, May *et al.*, 1997, Balasubramanian *et al.*, 1998, Eng *et al.*, 1998, Mulvihill *et al.*, 2000, Wong *et al.*, 2000). Table 1 shows the main components of the contractile ring and other elements involved in *S. pombe* cytokinesis. CAR assembly involves three genetically separable steps: establishment of a cortical network of CAR proteins, its lateral condensation, and finally, the formation of a homogeneous CAR (Hachet & Simanis, 2008).

### 4.3. Ring contraction

Mid1p leaves the ring before ring contraction starts, suggesting that it might have an inhibitory role in ring contraction and, hence, must be removed before cell division. An increase in type-II myosin activity is also a likely candidate for initiating ring contraction (see Wolfe & Gould, 2005 for review). The UCS-domain-containing protein Rng3p, which is required for type-II myosin function *in vivo*, stimulates the F-actin filament gliding activity of myosin in a reconstituted assay, thus raising the possibility that Rng3p regulation might be involved in stimulating ring contraction (Wong *et al.*, 2000, Lord & Pollard, 2004).

The contraction of the medially-placed F-actin ring, which in turn directs the synthesis of the septum, depends on a complex of proteins that constitute a signaling route called SIN (from Septum Initiation Network. See reviews Simanis, 2003, Krapp *et al.*, 2004). The SIN complex is composed of several proteins (figure 9); the GTPase Spg1p (Schmidt *et al.*, 1997), which in its GTP-bound form binds the protein kinase Cdc7p (Fankhauser & Simanis, 1994), initiates a cascade of signal transduction which requires two other kinases, Sid1p and Sid2p, and their associated subunits, Cdc14p and Mob1p, respectively (Fankhauser & Simanis, 1993, Sparks *et al.*, 1999, Guertin *et al.*, 2000, Hou *et al.*, 2000). The GTPase activating protein (GAP) for Spg1p comprises two proteins: Cdc16p and Byr4p (Furge *et al.*, 1998). To date, no guanine-nucleotide-exchange factor (GEF) specific for Spg1p has been identified in screens for SIN mutants. It is thought that spontaneous exchange of GTP for GDP on Spg1p is sufficient for regulation of the signaling status of Spg1p, without the requirement for a specific GEF (Simanis, 2003). The protein

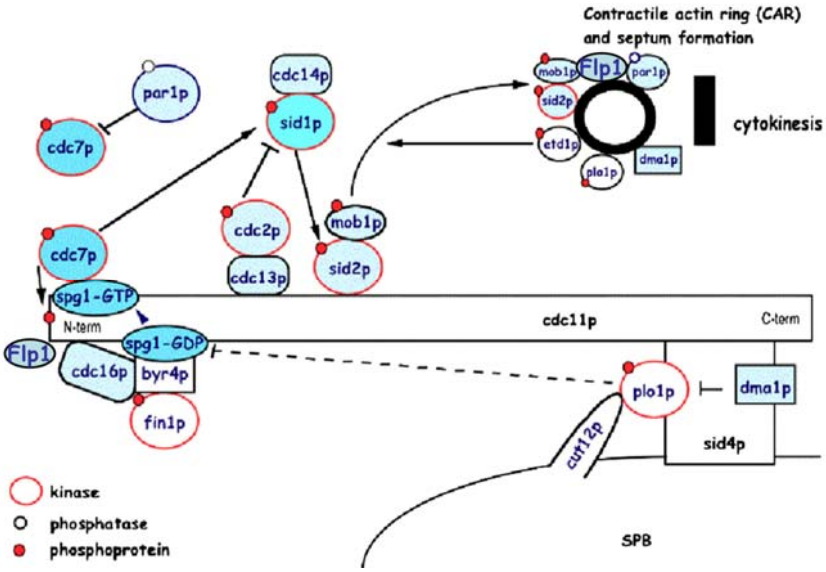



**Figure 9. Assembly of the SIN proteins at the SPB and CAR**. The SIN components localizes to the SPB by docking to the scaffold proteins Sid4p and Cdc11p. The arrow represents activation and the T-shape line inhibition. Kinases are surrounded by red circles. Phosphoproteins are specified by a red dot and phosphatases by a white dot. Taken from Bedhomme *et al*., 2008.

kinase Plo1p is also involved in SIN activation (Mulvihill *et al.*, 1999, Tanaka *et al.*, 2001).

Most of the SIN components assemble into a large protein complex that localizes to the SPB (figure 9).The Sid4p and Cdc11p SIN components function as docking proteins for the localization of SIN components to the SPB (Morrell *et al.*, 2004). In dividing cells, the two spindle poles are not equivalent, with an old and a new SPB. This ensures that the cell machinery is correctly oriented (Simanis, 2003). Basically, the SIN complex-signaling cascade is started by the union of active (GTP-bound) Spg1p to the Cdc7p kinase. Then, this complex, which binds to the adaptor protein Cdc11p at the SPB, is capable of activating Sid1p (together with its regulator Cdc14p), which in turn activates the Sid2p-Mob1p complex. Later, Sid2p-Mob1p leaves the SPB and localize to the CAR (Krapp *et al.*, 2004). It has been shown that Etd1p is one of the final effectors of the SIN pathway, promoting the contraction of the ring through its interaction with Cdc15p (Daga *et al.*, 2005). It is possible that there are other effectors of the Sid2p-Mob1p complex that contribute to ring contraction. One of these targets might be the type-II myosins Myo2p and/or Myo3p, since it has been described that Myo2p is not properly localized in some SIN-defective mutants (Mulvihill *et al.*, 2001). Another possible effector of the SIN pathway is the β(1,3)glucan synthase Bgs1/Cps1p, which is required for the synthesis of the primary septum (Cortes *et al.*, 2007).

SIN signaling is negatively regulated by Dma1p (Murone & Simanis, 1996, Guertin *et al.*, 2002), so a strong expression of Dma1 inhibits septum formation. It has been suggested that Dma1p prevents inappropriate localization of Plo1p. It interacts with the scaffold protein Sid4p, which is thought to anchor it at the spindle pole body (Guertin *et al.*, 2002). Dma1p also appears at the CAR during anaphase; its role and anchors there are unknown. Two other putative regulators of SIN signaling, Zfs1p (Beltraminelli *et al.*, 1999) and Scw1p (Karagiannis *et al.*, 2002, Jin & McCollum, 2003), have also been identified. Zfs1p is a zinc-finger protein, whereas Scw1p is an RNA-binding protein. Loss of either protein reduces, but does not eliminate, the requirement for SIN signaling. Their mode of action is unclear.

A number of protein phosphatases are also involved in regulating the SIN complex, although it is not clear what their relevant substrates are: Par1p-PP2A which acts as a negative regulator (Le Goff *et al.*, 2001), calcineurin encoded by the gene *ppb1*$^{+}$ (Lu *et al.*, 2002), and Flp1/Clp1p, which is a component of the cytokinesis checkpoint and mediates the coordination between the nuclear cycle and cytokinesis. The SIN is required to keep CDK (Cdc2p) activity low in order to prevent entry in a next cell cycle. This function is mediated by the phosphatase Flp1/Clp1p which is regulated by sequestration inside the nucleolus during interphase (see Bedhomme *et al.*, 2008 as a review).

### 4.4. Addition of new plasma membrane

Membrane remodeling is inherent to the process of cell division. The single plasma membrane that surrounds the mother cell must be subdivided into two separable domains at the end of cytokinesis. In addition, under most conditions, new membrane is generated to accommodate the increased surface area of the two daughter cells. This process of insertion of new plasma membrane can also provide certain proteins or other components relevant to the process of cytokinesis. In fission yeast, secretion of new membranes and assembly of the division septum occur in concert with constriction of the actomyosin ring (see review Balasubramanian *et al.*, 2004).

Recently, the importance of sterol-rich membrane domains, known as *lipid rafts*, in the process of cell division has been described. The lipid rafts membrane domains are rich in cholesterol or ergosterol and sphingolipids and are resistant to extraction with cold non-ionic detergents. Lipid rafts are thought to compartmentalize the plasma membrane and to have important roles in cell signaling, polarity and sorting (Mukherjee & Maxfield, 2004). In the fission yeast *S. pombe*, sterols are enriched in the plasma membrane at the growing cell tips and at the site of cytokinesis. The distribution of sterols is regulated in a cell cycle-dependent manner and requires a functional secretory pathway (Wachtler *et al.*, 2003, Wachtler & Balasubramanian, 2006).

The formation of these membrane domains requires Cdc15p, an essential CAR protein that associates with lipid rafts and organizes them at the cytokinesis cleavage site. *cdc15* mutants exhibit spiral-shaped membrane domains. *cdc15*$^{+}$ over-expression in interphase cells induces abnormal membrane domain formation in an actin-independent manner (Takeda *et al.*, 2004). Myo1p, which is also required for proper organization of these membrane domains (Takeda & Chang, 2005), is recruited to the site of cell division by Cdc15p through a direct interaction (Carnahan & Gould, 2003). It has been shown that the integrity of these domains is crucial for positioning and/or maintenance of the actomyosin ring, as well as for the stability of some integral membrane proteins (Wachtler *et al.*, 2003). Therefore, lipid rafts could have a space-restriction function for the localization of the division machinery in the cell equatorial zone. Thus, these membrane microdomains would facilitate the addition of new membrane to this zone or prevent the diffusion of proteins along the membrane (Wachtler & Balasubramanian, 2006).

### 4.5. Formation of the division septum

The position of the CAR anticipates the site of septum formation. Septation begins at the end of anaphase, when the spindle begins to break down, and is accompanied by the accumulation of actin patches at the septation area and the contraction of the ring at the septum's leading edge (Marks, 1985, Jochova *et al.*, 1991). The primary septum grows inwards from the cell cortex (Johnson *et al.*, 1973).

In fission yeast the primary septum, which is rich in linear β(1,3)glucan and can be visualized by Calcofluor staining (Horisberger & Rouvet-Vauthey, 1985, Humbel *et al.*, 2001, Cortes *et al.*, 2007), is laid down in a centripetal fashion until it completely closes and compartmentalizes the two daughter cells. Cps1p is an integral membrane protein that localizes to the division site and is the putative catalytic subunit of a β(1,3)glucan synthase essential for septum assembly (Ishiguro *et al.*, 1997, Le Goff *et al.*, 1999, Liu *et al.*, 1999, Cortes, 2002, Cortes *et al.*, 2007). After primary septum is complete, each daughter cell contributes to synthesize cell wall material to its own side of the primary septum, building the secondary septum. Electron microscopy studies have shown that while the primary septum mainly contains linear β(1,3)glucan the secondary septa contain α(1,3)glucan, (1,6)branched β(1,3)glucan, β(1,6)glucan and galactomannans (Horisberger & Rouvet-Vauthey, 1985, Humbel *et al.*, 2001, Sugawara *et al.*, 2003, Durán & Pérez, 2004). For more detail see section 3.4.1. Bgs4p seems to exert its role in the synthesis of the secondary septum (Cortes *et al.*, 2005). The potential α(1,3)glucan synthases Mok1/Ags1p probably contributes to the synthesis of the secondary septa because it is essential for cell viability during the vegetative growth and is involved in cytokinesis (Hochstenbach *et al.*, 1998, Katayama *et al.*, 1999). Other *mok* genes are involved in the process of sporulation (Garcia *et al.*, 2006).

Genetic studies indicate that activation of the SIN pathway regulates Cps1p, the β(1,3)glucan synthase subunit essential for the assembly of the division septum (Le Goff *et al.*, 1999, Liu *et al.*, 2000b).

### 4.6. Septum degradation and cell separation

At the end of cytokinesis, the multilayered division septum must be cleaved to release the two daughter cells. The separation of the sister cells requires two degradative processes: erosion of the surrounding cylinder of cell wall at its junction with the septum and dissolution of the primary septum (Robinow and Hyams, 1989). Cell separation requires enzymatic hydrolysis of the primary septum in a process that is mediated by the β-glucanase and α-glucanase encoded by *eng1*$^+$ and *agn1*$^+$, respectively (Martin-Cuadrado *et al.*, 2003, Garcia *et al.*, 2005). These enzymes are required to achieve efficient cell separation because Eng1p is necessary for controlled dissolution of the primary septum (Martin-Cuadrado *et al.*, 2003) and Agn1p is involved in the degradation of the septum edging (Dekker *et al.*, 2004). There are other genes in the genome of *S. pombe* that show similarity to glucanases and might be involved in the cell separation and/or other morphogenetic processes that require the degradation of the cell wall.

The exocyst is an octameric protein complex present in many organisms and is involved in tethering vesicles from the Golgi apparatus to specific sites on the plasma membrane (TerBush *et al.*, 1996, Hsu *et al.*, 1999, Wang *et al.*, 2002, Hsu *et al.*, 2004). In *S. pombe*, this complex is necessary for cell separation because it is required for the delivery of the hydrolytic proteins Eng1p and Agn1p to the septum (Wang *et al.*, 2002, Martin-Cuadrado *et al.*, 2005). Septins and Mid2p are also required for Eng1p and Agn1p localization to the septum (Martin-Cuadrado *et al.*, 2005). Mid2p is required to maintain the stability of the septin ring, and *mid2Δ* mutants show a phenotype very similar to that of septins mutants (Berlin *et al.*, 2003, Tasto *et al.*, 2003). Indeed, Mid2p is the main factor required for septins to coalescence and to organize into a ring (An *et al.*, 2004). In wild-type cells, the septin ring (stabilized by Mid2) acts as a positional marker to direct the targeting of exocyst-mediated secretory vesicles to the region of the cell where the membrane and the septum contact each other. This would allow the release of the cargo proteins (including the hydrolytic enzymes Eng1p and Agn1p) into the septum edging as a ring that completely surrounds the septum. Once this ring has been assembled, cell separation would proceed in a centripetal manner, from the septum edging toward its center (Martin-Cuadrado *et al.*, 2005).

Finally, to achieve efficient cell separation and to avoid cell lysis, a perfect temporal and spatial regulation of biosynthetic and hydrolytic activities is required, and these processes must also be perfectly coordinated with other events occurring in the cell cycle.

## 5. Stress and cell integrity in *S. pombe*

The MAPK (Mitogen-Activated Protein Kinase) pathways are signal transduction pathways that regulate many cellular processes in eukaryotic cells. These pathways respond to certain stimulus and transmit a signal to the cell interior through the sequential phosphorylation of three protein kinases; initially, a MAPKKK (MAPK kinase kinase) is activated by phosphorylation and phosphorylates a MAPKK (MAPK kinase), which in turn phosphorylates a MAPK (Marshall, 1995, Waskiewicz & Cooper, 1995).

While in the budding yeast *S. cerevisiae*, several MAPK pathways have been characterized (Hohmann, 2002), in *S. pombe* only three of them have been identified. One of these pathways transmit the signal in response to pheromones and requires the Byr2p, Byr1p and Spk1p kinases (Nielsen, 2004); the stress response pathway is transmitted through the Wak1p and Win1p, Wis1p and Sty1/Spc1p kinases (Toda *et al.*, 1991, Shiozaki & Russell, 1995), and the so-called cell integrity pathway involves the Mkh1p, Pek1/Skh1p and Spm1/Pmk1p kinases (Toda *et al.*, 1996, Sengar *et al.*, 1997, Zaitsevskaya-Carter & Cooper, 1997, Sugiura *et al.*, 1999, Loewith *et al.*, 2000).

Initially, it was described that disruption of any of the members of the cell integrity pathway led to a series of phenotypes including hypersensitivity to β(1,3)glucanases, abnormal growth rate in response to hyperosmotic conditions, and altered cytokinesis in

response to stress (Toda *et al.*, 1996, Zaitsevskaya-Carter & Cooper, 1997). Later, it was found that this pathway is activated in response to many stimuli: hyper and hypo-osmotic stress, glucose deprivation, cell wall damage, high temperatures, and oxidative stress (Madrid *et al.*, 2006). A characteristic of the mutants defective in this pathway is their ability to suppress the absence of Ppb1p (calcineurin). In *S. pombe*, the absence of Ppb1p, or its inactivation using the immunosuppresant FK506, leads to hypersensitivity to chloride ions. This hypersensitivity is suppressed when a component of the cell integrity pathway is deleted. Thus, the *vic* phenotype (viable in the presence of immunosuppressant and chloride ion) is a characteristic of the mutants in this pathway and, therefore, it can be used to identify new components of the pathway by analyzing the growth of a strain in the presence of FK506 and $MgCl_2$ (Sugiura *et al.*, 1998, Sugiura *et al.*, 1999).

Several genes involved in cell wall synthesis have been found to participate in this pathway (Ma *et al.*, 2006, Takada *et al.*, 2007, Barba *et al.*, 2008, Garcia *et al.*, 2009). Ma *et al* have demonstrated a relationship between Rho2p and Pck2p with the protein kinase C–MAPK cell integrity pathway. Pck2p physically interacts with Mkh1p and overexpression of Rho2p and Pck2p increases Pmk1p/Spm1p phosphorylation, suggesting that Rho2p activates the cell integrity pathway through Pck2p (Ma *et al.*, 2006).

# Antecedents & Objective

*Schizosaccharomyces pombe* has no detectable amounts of chitin in its cell wall. However, when the genome of this organism was sequenced several proteins with similarity to chitin synthases (CS) and their regulators were found (information about the *S. pombe* sequences can be found in http://www.sanger.ac.uk/Projects/S_pombe/). *A priori*, it seemed possible that the study of these proteins could allow unveiling their basic function, which in *Saccharomyces* would have been adapted to the regulation of chitin synthesis.

Chs1p, a protein with similarity to CS enzymes from other fungi turned out to be a *bona fide* CS enzyme required for proper maturation of the ascospores (Arellano *et al.*, 2000) Chs2p, a CS-like protein devoid of the amino acids considered to be the catalytic domain (QRRRW) is not required for cell wall biosynthesis, but plays a role in cytokinesis because it is a linker between the plasma membrane and the myosin component of the contractile actomyosin ring (Martin-Garcia *et al.*, 2003, Martin-Garcia & Valdivieso, 2006). In parallel, a similar role was found for the *S. cerevisiae* Chs2 protein (VerPlank & Li, 2005), showing that both proteins share a function in stabilizing the ring during contraction.

Chs5p is a Golgi protein required for the transport of the CS Chs3p to the plasma membrane (Santos *et al.*, 1997, Santos & Snyder, 1997). It is also necessary for the transport of Fus1p to the tip of the shmoos during mating, so a *chs5Δ* mutant exhibits a mating defect that is independent of the role of Chs5p in chitin synthesis (Santos *et al.*, 1997, Santos, 2003). *S. pombe* Cfr1 protein shares sequence similarity with Chs5p. Deletion of the *cfr1*$^+$ gene does not affect cell wall synthesis but results in a mating defect similar to that of the *S. cerevisiae chs5Δ* mutants (Cartagena-Lirola *et al.*, 2006).

The above results reinforced the idea that some of the *CHS*-like genes in *S. pombe* share some function with the *S. cerevisiae CHS* genes. Chs4p belongs to a family of proteins, present in all organisms except for *Archaea*, characterized for the presence of tandem repeats of SEL1 domains. SEL1 domains are hypothetically involved in protein-protein interactions and are present in multiprotein complexes that participate in different cellular processes (Mittl & Schneider-Brachert, 2006). Chs4p seems to regulate the CS Chs3p in different ways: i) Chs4p anchors Chs3p to the septin ring through an intermediate protein termed Bni4p, ii) Chs4p regulates the biochemical activity of Chs3p, and iii) Chs4p regulates Chs3p stability at the plasma membrane (DeMarini, 1997, Trilla *et al.*, 1997, Ono N, 2000, Grabinska *et al.*, 2007, Reyes *et al.*, 2007). In *S. pombe*, there is no chitin synthesis during vegetative growth, there is no Bni4p homologue, and the septins are involved in cell separation but not in septum synthesis (Martin-Cuadrado *et al.*, 2005). Chs2p, with similarity to CS enzymes, plays a role in cytokinesis but not in cell wall synthesis and, finally, primary septum is composed of β-glucan in the fission yeast.

In the *S. pombe* genome there are 4 protein sequences with similarity to Chs4p, which we have named Cfh1p to Cfh4p (from Chs four homologues). All of them have several SEL1 repeats (figure 10).

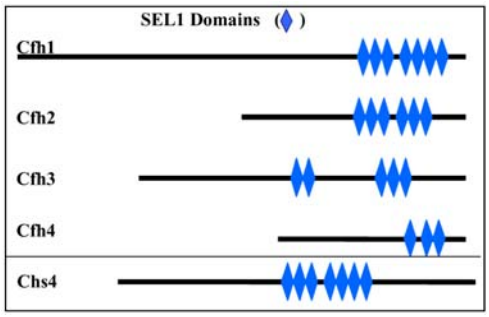



**Figure 10. Distribution of SEL1 domains in Chs4-related proteins from *S. pombe*.**

Deletion of each *cfh*$^+$ gene, or of all of them, gives no obvious phenotype (Matsuo *et al.*, 2004 and our unpublished results). Overexpression of *cfh1*$^+$ and *cfh4*$^+$ leads to a loss in polarity (see figure 11), overexpression of *cfh2*$^+$ produces cells with heterogeneous morphology (figure 11) and *cfh3*$^+$ overexpression leads to a defect in cytokinesis (figure 11 and Matsuo, *et al*. 2004).

The phenotype produced by the overexpression of *cfh3*$^+$ reminds that of the overexpression of *chs2*$^+$ (figure 11, Matsuo *et al.*, 2004, and Martin-Garcia & Valdivieso, 2006), suggesting a role of Cfh3p in cytokinesis. Matsuo *et al*. described that in the absence of *cfh3*$^+$ Chs2-GFP is observed at its normal localization (the contractile ring) and in cytoplasmic dots so they

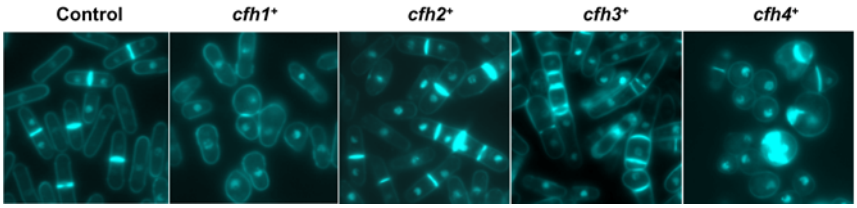



**Figure 11. Phenotype of cells overexpressing the *cfh*$^{+}$ genes.** Cells were stained with Hoechst and photographed.

concluded that Cfh3p is required for Chs2p localization. However, throughout our work with Chs2p we had observed that such localization could occur also in the WT strain, depending on the culture conditions.

From the information described above several questions arise:

Are Cfh proteins regulators of Chs2p during cytokinesis?.

Are Cfh proteins related to septins and cell separation?.

Are Cfh proteins regulators of the glucan synthases?.

Has each Cfh protein undertaken each one of these roles?.

Are there complexes containing SEL1-proteins regulating morphogenesis in different organisms?.

## OBJECTIVE

**The objective of the present work was to study the role of Cfh3p in cell wall synthesis and in morphogenesis in *Schizosaccharomyces pombe*.**

# Results

## 1. Initial characterization of *cfh3* mutants

As it has been explained in the Antecedents section, it seemed that *cfh3*$^+$ could have a function in cytokinesis, so we wished to determine which this function might be. To do so, we analyzed the phenotype of null and overexpression mutants.

### 1.1. *cfh3Δ* null mutant

In order to understand the function of *cfh3*$^+$, a null mutant was constructed as explained in "Materials and Methods". When we observed the cells from the null mutant under the phase contrast microscope we did not find any apparent difference with the cells from the WT strain. Also, the doubling time for the WT and the mutant strains incubated in rich or minimal medium at different temperatures was the same (not shown).

### 1.2. Overexpression strain

To overexpress the *cfh3*$^+$ gene, we constructed a plasmid using the pREP3X vector, which carried the *nmt1*$^+$ promoter (derepressible in the absence of thiamine) and included the *cfh3*$^+$ ORF and terminator. A WT control strain (HVP458, see Table 3 in the appendix) was transformed with this plasmid and the phenotype produced by the overexpression of *cfh3*$^+$ was analyzed after several hours of derepression. After 24 hours in the absence of thiamine, cells were collected and stained with Hoechst. As shown in the figure 12, cells exhibited several problems in cytokinesis such as branches (diamonds), two septa delimiting anucleated cell compartments (dots), multiple septa at the cell midzone (asterisks), and nuclei trapped by the septa (arrow-heads). This result suggested that *cfh3*$^+$ had a function in cytokinesis.

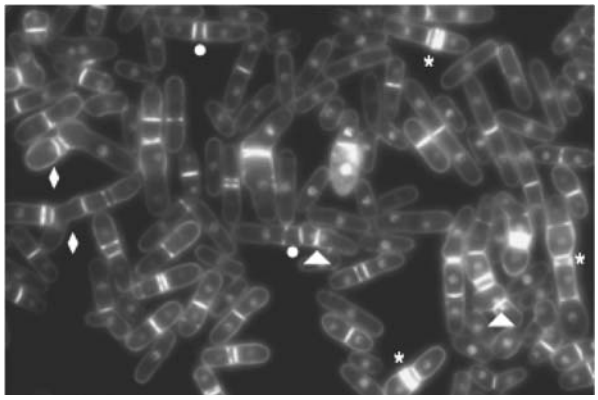


**Figure 12. Phenotype produced by *cfh3*$^+$ overexpression.** Diamonds mark branched cells, dots mark anucleated cell compartments, asterisks mark multiple septa, and arrow-heads mark nuclei trapped by septa.

## 2. Localization of Cfh3 protein

In order to know which the localization of Cfh3p was, we constructed an integrative plasmid bearing the *cfh3*$^+$ gene tagged with the GFP at the amino terminus under the control of its own promoter. In this way, we could observe the Cfh3p in living cells by following the localization of the GFP. With the aim of knowing the dynamics of Cfh3p localization with respect to the nuclear division, a strain carrying a GFP-tagged histone and the GFP-Cfh3 fusion protein was constructed. We found that Cfh3p localized at the division zone of those cells that were undergoing cytokinesis and that the fluorescent signal contracted during cell division, suggesting a relationship with the contractile acto-myosin ring (figure 13 A). In addition, we observed Cfh3p at the cell poles. Apparently, Cfh3p disappeared from the poles and localized at the midzone at the beginning of nuclear division (figure 13 A).

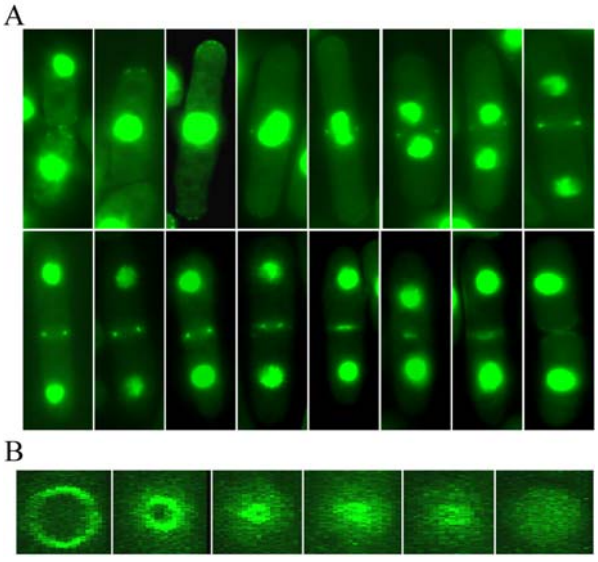



**Figure 13. Cfh3p localization.** (A) Cells carrying GFP-fused Cfh3p and histone Hht2p were photographed under a conventional fluorescence microscope. (B) Cells carrying GFP-Cfh3p were observed under a confocal microscope. Pictures were taken every 7 minutes.

To follow the behavior of the protein at the division site, we observed the GFP-Cfh3 protein under the confocal microscope (figure 13 B). We observed that Cfh3p forms a ring that leaves a faint signal as a plate when the ring closes. The *ring* pattern is typical of proteins associated to the CAR and the *plate* pattern is similar to that of the Bgs1glucan synthase (a transmembrane protein) and to that of some GTPases (which are membrane-associated proteins). Cfh3p has a potential prenylation site, so it is possible that it associates with the membrane. This result is in agreement with Cfh3p being involved in the process of cytokinesis, and suggested that Cfh3p could be associated to both, the CAR and the plasma membrane.

## 3. Relationship between *cfh3*$^+$ and other genes involved in cytokinesis

With the purpose of verifying if the raised hypothesis was correct, and in order to find out in what moment or stage of cytokinesis Cfh3p exerts its function, different experimental approaches were undertaken. They are detailed below.

### 3.1. Localization of proteins involved in cytokinesis in cells overexpressing *cfh3*$^+$

This strategy would allow us to find out which proteins were abnormally distributed in cells overexpressing *cfh3*$^+$ and, therefore, which alterations were associated with the multi-septation phenotype observed in the WT strain overexpressing this gene. In this way, physical interactions and functional relationship between the Cfh3p and other proteins involved in cell division could be envisaged. To undertake this approach, strains carrying GFP-tagged proteins (except for actin, see below) involved in different stages cytokinesis and overexpressing *cfh3*$^+$ were observed under the fluorescence microscope. We analyzed the distribution of CAR components (actin, the myosin light chains Cdc4p and Rlc1p, and Cdc15p), a protein that links the CAR to the plasma membrane (Chs2p), and proteins involved in cell separation (the septin Spn3p and the glucanases Agn1p and Eng1p). The results are detailed below.

#### 3.1.1. Localization of actin in cells overexpressing *cfh3*$^+$

Actin takes part in the formation of the CAR and consequently in cytokinesis. Actin is necessary not only for the assembly of the ring, but also for the correct positioning of many components of the CAR. Additionally, actin can be observed at the cell poles. In order to see the localization of actin in cells overexpressing *cfh3*$^+$, actin was stained with Rhodamine-Phalloidin and the cell wall was stained with Calcofluor. As shown in the figure 14, in the WT strain actin localized at the CAR and in the form of patches at the cell poles and equator. In the cells overexpressing *cfh3*$^+$, actin patches accumulated all around the cell, and formed multiple rings in about 10% of the cells undergoing cytokinesis. This result shows that *cfh3*$^+$ overexpression produces alterations in the distribution of actin.

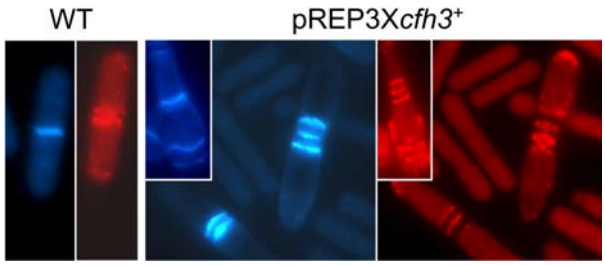



**Figure 14. *cfh3*$^+$ overexpression disturbs actin localization**. Calcofluor (left panels) and rhodamine-phalloidin (right panels) staining of wild-type cells or cells overexpressing *cfh3*$^+$.

#### 3.1.2. Localization of myosin components in cells overexpressing *cfh3*$^+$

In *S. pombe*, type-II myosins are in a complex of three proteins including a heavy chain (Myo2p or Myo3p), an essential light chain (Cdc4p), and a regulatory chain (Rlc1p). These proteins are required for CAR assembly and maturation. We analyzed the localization of myosin components in cells overexpressing *cfh3*$^+$. Cdc4p, the essential light chain of the myosin, is associated to the heavy chains Myo2/Myo3 and localizes early at the CAR. In the WT strain, and in agreement with previous results (McCollum *et al.*, 1995), Cdc4p was only observed at the equator of the cells forming a ring that contracted until it formed a dot and disassembled after the septum was synthesized (figure 15). When cells overexpressed *cfh3*$^+$, 60% of the cells (n=500) showed aberrant Cdc4p localizations,

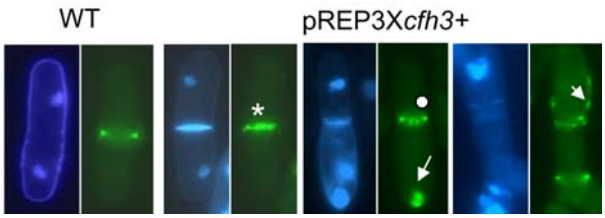



**Figure 15. *cfh3*$^+$ overexpression disturbs myosins at the CAR**. Hoechst (left panels) and GFP (right panels) fluorescence of wild-type cells or cells overexpressing *cfh3*$^+$. The asterisk marks a cell in which Cdc4p remains after the septum is complete, the dot marks a cell with two rings and the arrows mark cells in which Cdc4p accumulates at the cell cortex.

which included protein that remained at the midzone after the septum had been synthesized (20% of the cells; see asterisk in figure 15), multiple rings (70% of the cells; dot in figure 15), and protein localized at the cell cortex (10% of the cases; arrows in figure 15). Similar results were obtained for Rlc1p (results not shown). In conclusion, *cfh3*$^+$ overexpression produces alterations in the distribution of the type-II myosins at the CAR.

### 3.1.3. Localization of the Cdc15p in cells overexpressing *cfh3*$^+$

Cdc15p is one of the components of the CAR, and it is involved in the processes of polymerization and assembly of actin in this ring. Cdc15p can also be observed forming patches near the cellular poles during interphase, although it has been described that Cdc15p does not co-localize totally with the actin patches (Fankhauser *et al.*, 1995). In the cells that are undergoing division, Cdc15p disappears from the poles and localizes at the medial region of the cell, forming a ring in the zone where the nucleus is placed. The ring, then, contracts towards the cellular interior and, finally, disassembles. After the disassembling of the Cdc15p ring, the protein is observed in the form of patches in the zone of the old pole of the cells (Carnahan & Gould, 2003). When *cfh3*$^+$ was overexpressed, an aberrant distribution of this protein was observed in 80% of the cells (n= 500), including accumulation of the protein at the cell cortex as dots or thread-like structures (50% of the cells; see arrows in figure 16), multiple rings (20% of the cells, see the dot in figure 16) or asymmetric rings (30% of the cells; see the asterisk in figure 16). The results showed that *cfh3*$^+$ overexpression produces alterations in the distribution of Cdc15p.

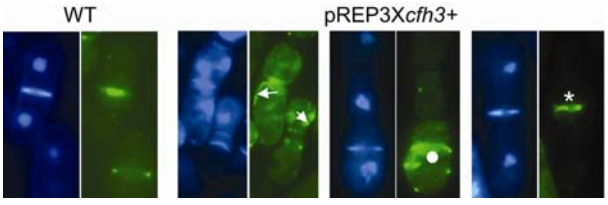



**Figure 16. *cfh3*$^+$ overexpression disturbs the distribution of Cdc15p**. Hoechst (left panels) and GFP (right panels) fluorescence of wild-type cells or cells overexpressing *cfh3*$^+$. The arrows mark cells in which Cdc15p accumulates at the cell cortex, the dot marks a cell with two rings and the asterisk marks an asymmetric ring.

### 3.1.4. Localization of the Chs2p in cell overexpressing *cfh3*$^+$

Chs2p is a transmembrane protein that associates with the CAR (Matsuo *et al.*, 2004, Martin-Garcia & Valdivieso, 2006). Chs2p localizes at the CAR and is required for the stability of the ring. However, when cells overexpressed *cfh3*$^+$ it was possible to observe septating cells with no Chs2p signal (20% of the cells, n=500; see arrows in figure 17) or cells in which the signal at the ring was asymmetric (35% of the cells, n= 500; asterisk in figure 17). Therefore, *cfh3*$^+$ overexpression produces alterations in the distribution of Chs2p.

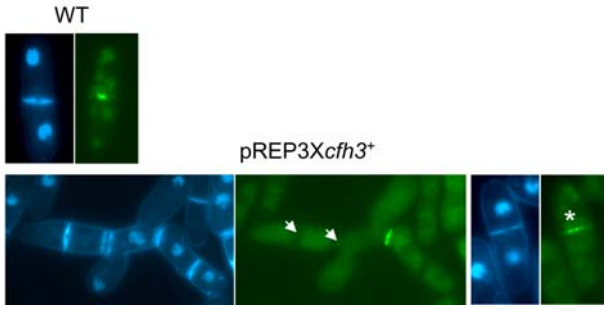



**Figure 17. *cfh3*$^+$ overexpression disturbs the distribution of Chs2p**. Hoechst (left panels) and GFP (rigt panels) fluorescence of wild-type cells or cells overexpressing *cfh3*$^+$. The arrows mark cells in which Chs2p is not observed in septating cells and the asterisk marks an asymmetric ring.

### 3.1.5. Localization of septins in cells overexpressing *cfh3*$^+$

Septins are a family of proteins that bind GTP and are conserved from yeasts to animal cells. They are involved in cellular separation, polarity, and secretion and membrane deposition. In *S. pombe*, septins

are involved in the localization of glucanases and therefore in cell separation. Septins localize as a ring that does not contract during cytokinesis at the mid-zone of WT cells (An *et al.*, 2004). In cells overexpressing *cfh3*$^+$, 80% of the cells (n=500) carrying a GFP-tagged Spn3p showed fluorescent patches at the cell periphery or in the cytoplasm (65% of the cells; arrows in figure 18) or a signal that was at the leading edge of the growing septum (15% of the cases; lower panels in figure 18). The results showed that *cfh3*$^+$ overexpression produces alterations in the distribution of the Spn3p.

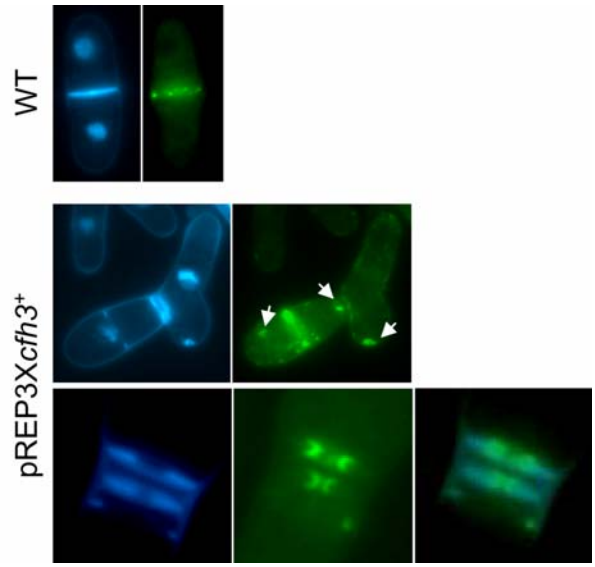



**Figure 18. *cfh3*$^+$ overexpression disturbs the distribution of septins**. Hoechst (left panels) and GFP (right panels) fluorescence of wild-type cells or cells overexpressing *cfh3*$^+$. The arrows mark cells in which Spn3 accumulates at the cell cortex. The panels on the bottom are enlarged to allow a view of the septal area of a cell with multiple septa; Hoechst, GFP and the merge images are shown.

### 3.1.6. Localization of glucanases in cell overexpressing *cfh3*$^+$

Agn1p and Eng1p glucanases are enzymes located in the zone of division and play a role in cell separation (Alonso-Nunez *et al.*, 2005, Martin-Cuadrado *et al.*, 2005). We wanted to know whether overexpression of *cfh3*$^+$ had any effect in the localization of glucanases. To do this, we observed cells bearing GFP-fused Agn1p or Eng1p that overexpressed *cfh3*$^+$. We found that 70% of the cells (n=500) showing at least one septum did not exhibit the Agn1-GFP or the Eng1-GFP signal in some of them (figure 19 and results not shown). These results indicated that *cfh3*$^+$ overexpression produces alterations in the distribution of the glucanases.

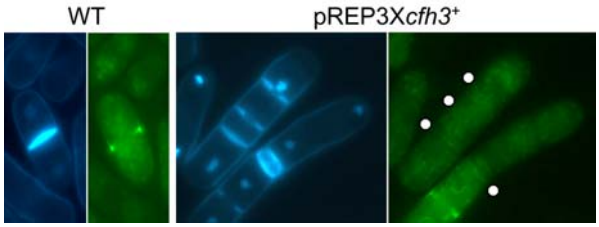



**Figure 19. *cfh3*$^+$ overexpression disturbs the distribution of glucanases**. Hoechst (left panels) and GFP (right panels) fluorescence of wild-type cells or cells overexpressing *cfh3*$^+$. The dots mark septa in which Agn1 cannot be observed or has a weak fluorescence.

### 3.1.7. Localization of Bgs1p in cell overexpressing *cfh3*$^+$

Bgs1p is a β-glucan synthase that is required for the synthesis of linear β-glucan. Bgs1p is a transmembrane protein and in the WT strain localizes at the septum and the cell poles (Cortes, 2002). We wanted to know whether overexpression of *cfh3*$^+$ had any effect in the localization of the Bgs1p. Cells bearing a GFP-fused Bgs1 protein and overexpressing *cfh3*$^+$ showed an aberrant localization of Bgs1p. In those cells showing a mild overexpression phenotype, Bgs1p localized in the poles but extended to the lateral membrane. In those cells showing a strong phenotype, Bgs1p localized at the membrane all around the cells (figure 20). In conclusion, *cfh3*$^+$ overexpression produces alterations in the distribution of the Bgs1p.

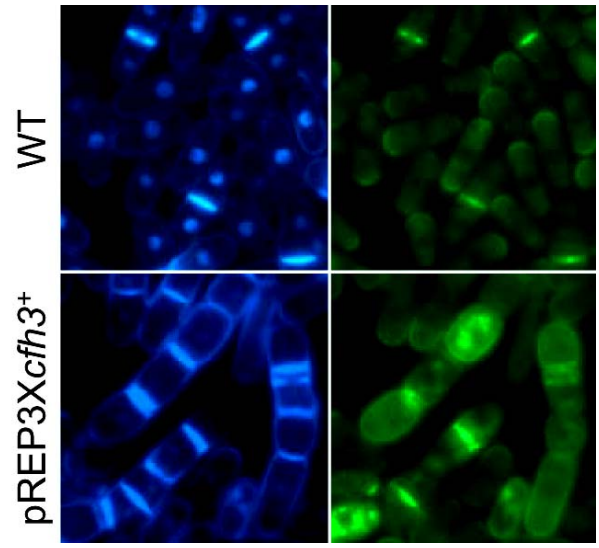



**Figure 20. *cfh3*$^+$ overexpression disturbs the distribution of Bgs1p**. Hoechst (left panels) and GFP-Bgs1 fluorescence (right panels) of wild-type cells or cells overexpressing *cfh3*$^+$.

Collectively, all the results described above show that a deregulated amount of Cfh3p interferes with different stages of cytokinesis and suggest that Cfh3p might be a component of a multiprotein complex.

### 3.2. Phenotype of *cfh3*$^+$ overexpression in mutants with an abnormal cytokinesis

In order to get information about the proteins that interact with Cfh3p we analyzed the phenotype of different cytokinesis mutants when they overexpress *cfh3*$^+$, the rationale being that in the absence of those proteins the overexpression phenotype could not be produced. Certain mutants affected in different stages of cytokinesis were chosen and *cfh3*$^+$ was overexpressed in them. We chose mutants affected in ring formation (*cdc15-140*), mutants affected in ring contraction and septum synthesis (the SIN *cdc11-119* and *cdc14-118* mutants, and *bgs1/cps1-191*) and mutant affected in cell separation (a quintuple *spn1Δ-spn5Δ* mutant). In order to observe the phenotype of overexpression it was needed to derepress *cfh3*$^+$ expression for a total of 24-25 hours. The strains were incubated at 25ºC for 16 hours and then shifted to the restrictive temperature (36ºC) for an additional 8 hours. The results obtained are described below.

#### 3.2.1. Phenotype of *cfh3*$^+$ overexpression in the *cdc15-140* mutant

Strain HVP532, carrying the *cdc15-140* mutation, was transformed with the pREP3X*cfh3*$^+$ plasmid. At permissive temperature (25ºC), and in the presence of thiamine, *cdc15-140* mutant exhibited a WT morphology (not shown). At the permissive temperature and in the absence of thiamine, the cells showed the phenotype of *cfh3*$^+$ overexpression (long multiseptated and branched cells; not shown). At the restrictive temperature (36ºC), and in the presence of thiamine, they showed the *cdc15-140* phenotype (long multinucleated cells with no septa. Figure 21). At the restrictive temperature and in the absence of thiamine, the cells showed the *cdc15-140* phenotype (long multinucleated cells without any septum; figure 21). This result indicates that phenotype of *cfh3*$^+$ overexpression requires the presence of a functional Cdc15 protein.

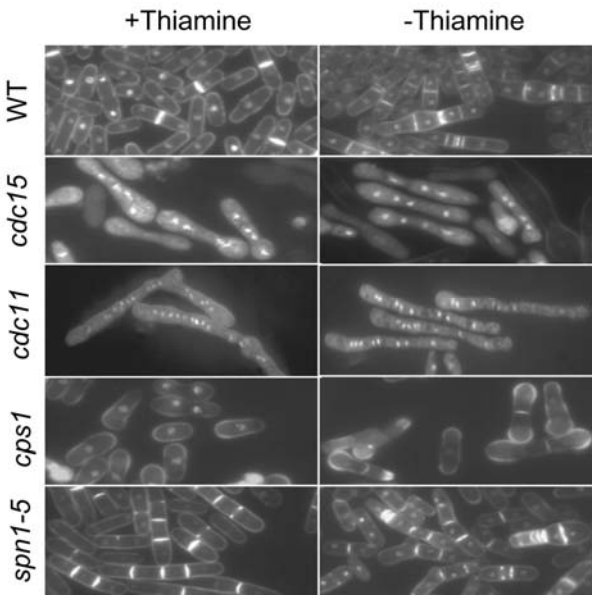



**Figure 21. Phenotype of the indicated strains overexpressing (-Thiamine) or not (+Thiamine) the *cfh3*$^+$ gene.** Cells were incubated at 36ºCand stained with Hoechst .

#### 3.2.2. Phenotype of *cfh3*$^+$ overexpression in the *cdc11-119* and *cdc14-118* mutants

As in the case of *cdc15-140*, at the restrictive temperature and in the absence of thiamine the *cdc11-119* cells carrying the pREP3X*cfh3*$^+$ plasmid showed the *cdc11-119* phenotype (long multinucleated cells with no septa, figure 21). We got the same results for the *cdc14-118* mutant (not shown). These results showed that the phenotype of *cfh3*$^+$ overexpression requires a functional SIN pathway.

#### 3.2.3. Phenotype of *cfh3*$^+$ overexpression in the *bgs1*/*cps1-191* mutant

When the *cps1-191* cells were cultured in minimal medium at 36ºC they did not arrest with two nuclei as synchronously as they do in YES medium. In any case, the cells did not exhibit any septa. When the *cfh3*$^+$ gene was overexpressed in these conditions, the characteristic multiseptation phenotype was not observed but 26% of the cells (n= 750) showed one septum (figure 21). This result could be interpreted as an indication of a partial dependence of the *cfh3*$^+$ overexpression phenotype on Bgs1p or as a partial suppression of the *cps1-191* thermosensitivity by a high dose of Cfh3p.

### 3.2.4. Phenotype of *cfh3*$^+$ overexpression in the absence of septins

The *spn1Δ-5Δ* mutant is devoid of the Spn1 to Spn5 septins. Under repressing conditions for the overexpression of *cfh3*$^+$, this mutant produced multi-septated cells that never showed more than one septa delimiting the cell bodies. However, in the absence of thiamine (*cfh3*$^+$ overexpression) some cells exhibited two or more proximal septa in-between two cell bodies, as it happened when *cfh3*$^+$ was overexpressed in the WT strain (figure 21). This result showed that the phenotype of *cfh3*$^+$ overexpression does not depend on septins 1 to 5.

Collectively, the results described in section 3.2. show that the phenotype of *cfh3*$^+$ overexpression requires an active SIN pathway and the Cdc15 protein.

### 3.3. Localization of proteins involved in cytokinesis in the *cfh3Δ* mutant

With this approach we aimed to get information about the consequences of the absence of Cfh3p. Strains carrying proteins involved in different stages of cytokinesis tagged with the GFP were crossed with *h*$^+$ or *h*$^-$ *cfh3Δ* cells. Then, strains carrying both, the *cfh3::KAN* deletion and each GFP-tagged protein were obtained by performing random spore analysis. We chose some proteins involved in CAR formation and stability (Cdc15-GFP, Cdc4-GFP and GFP-Chs2), a protein involved in septum synthesis (GFP-Bgs1), and proteins involved in cell separation (Spn3-GFP and Eng1-GFP). We observed that the mentioned proteins were localized properly in the *cfh3Δ* mutant (not shown), although about 10% of the Cdc15 and the Cdc4 rings were asymmetric or broken (see section 4). These results indicated that the Cfh3p is not required for the localization of proteins involved in cell division but that in the absence of this protein the rings are abnormal in some cells.

### 3.4. Localization of Cfh3p in mutants with an abnormal cytokinesis

With this approach we aimed to get information about the requirements for Cfh3p function and about the moment at which this function is exerted. To do so, we transformed the strains of interest with an integrative plasmid carrying the GFP-Cfh3 tagged protein under the control of its own promoter. We transformed different mutants affected in ring formation or stability (*cdc15-140*, *rlc1Δ* , *myo2E1 myo3::ADE2*, *cdc4-8*, and *chs2Δ*), mutants affected in the SIN signaling pathway (*cdc11-119* and *cdc16-116*), mutants affected in septum synthesis (*bgs1/cps1-191*), and mutants affected in cell separation (*spn3Δ* and *spn4Δ*). The results are detailed below:

### 3.4.1. Localization of Cfh3p in type II-myosin mutants

In order to study whether Cfh3p localization depended on the myosin component of the CAR, we analyzed its localization in the *myo2E1 myo3::ADE2, cdc4-8*, and *rlc1Δ* type II-myosin mutants. We found that after 3 hours of incubation at the restrictive temperature (36ºC for *myo2E1 myo3Δ* and *cdc4-8* and 20ºC for *rlc1Δ*), Cfh3p localized at the midzone of the cells in the *myo2-E1 myo3Δ* mutant, although not in a neat ring, probably because in these cells the ring is not properly formed (figure 22). In the case of the *cdc4-8* mutant, Cfh3p was observed in the midzone of the cell but it was restricted to a dot-like structure (not shown). When we analyzed Cdc15-GFP localization in the *cdc4-8* mutant, we observed a similar localization of this protein (not shown). This could be an indication that in these cells there are some ring proteins that stay at the midzone and that Cfh3p could be associated to these proteins. Respect to the *rlc1Δ* mutant, when this strain was incubated at 20ºC Cfh3p was observed in the midzone of the cell forming aberrant rings (not shown). In conclusion, although it is necessary that the ring is properly formed in the cells for Cfh3p to be observed as a neat ring, the localization of this protein at the cell equator does not require the myosin components of the CAR.

### 3.4.2. Localization of Cfh3p in the *cdc15-140* mutant

In the WT strain, a GFP-Cfh3p fused protein localizes at the cell midzone and poles. However, when the *cdc15-140* mutant was incubated for 3 hours at 36ºC, it was possible to detect Cfh3p at the cell poles, but not at the cell midzone (figure 22). This

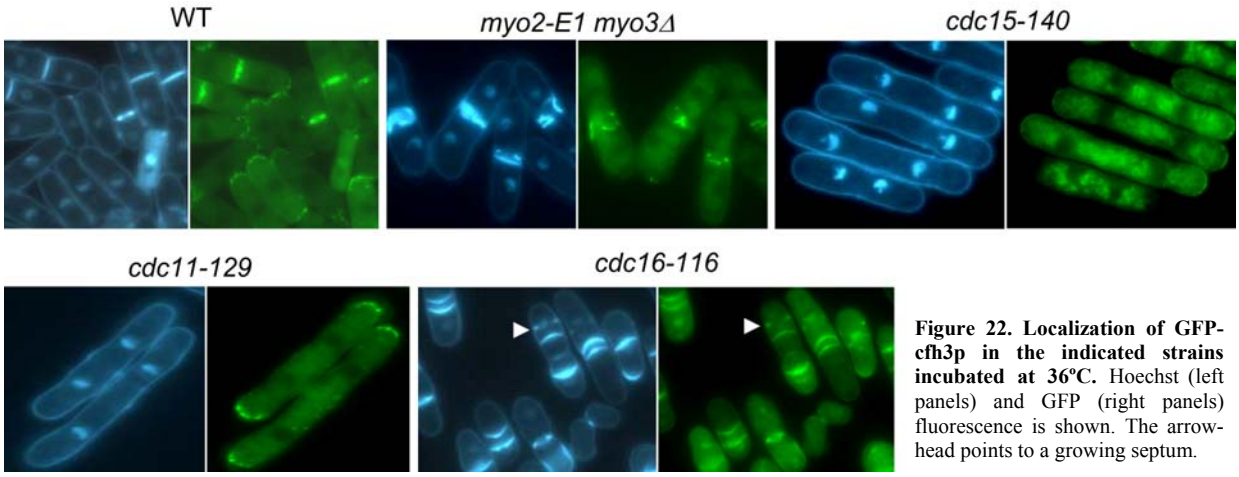



**Figure 22. Localization of GFP-cfh3p in the indicated strains incubated at 36ºC.** Hoechst (left panels) and GFP (right panels) fluorescence is shown. The arrow-head points to a growing septum.

result showed that localization of GFP-Cfh3p at the CAR depends on Cdc15p.

### 3.4.3. Localization of Cfh3p in SIN mutants

In order to study whether Cfh3p localization depends on the SIN pathway, we analyzed its localization in the *cdc11-119* and *cdc16-116* mutants. In the *cdc11-119* strain incubated at the restrictive temperature (36ºC) the SIN signal is inactive. Under these conditions the Cfh3 protein could be observed at the cell poles but not at the cell midzone (figure 22). In the *cdc16-116* mutant incubated at the restrictive temperature (36ºC), the SIN signal is hyperactive. We found that under these conditions Cfh3p could be observed at the inner side of the growing septa, as in the WT strain (arrow-head in figure 22), and that this protein could be observed at the medial region even in cells with complete septa (figure 22), a localization that is never observed in the WT strain. These results showed that the localization of Cfh3p at the ring depends on the activity of the SIN pathway.

As a summary, the facts that Cfh3p requires Cdc15p and the SIN pathway for its localization suggests that the function of this protein is required at a late moment during the ring assembly process and/or during ring contraction.

### 3.5. Analysis of genic interactions between *cfh3Δ* and mutants in genes involved in cytokinesis

In order to uncover the function that the Cfh3p might be performing under physiological conditions, we carried out a study of genetic interactions between *cfh3Δ* and mutants affected in several steps of cytokinesis. With this approach we would get information about the proteins that collaborate with Cfh3p. To do this, we constructed double mutants between *cfh3Δ* and mutants affected in different steps of cytokinesis by performing tetrad analysis or organized random spore analysis by micromanipulation. The latter method was used in those cases in which the morphology of the parental cells made it difficult to perform tetrad dissection (see materials and methods).

We chose mutants defective in ring formation or stability (*cps8-188*, *cdc15-140*, *myo2-E1, myo3Δ*, *myo2-E1, myo3Δ, cdc4-8*, and *chs2Δ*), in the SIN pathway (*cdc11-119* and *cdc14-118*), in the disassembly of the ring (*imp2Δ*), and in septum synthesis (*cps1-191*). These are ts mutants. Therefore, the analysis of the possible genetic interactions was realized by comparing the growth of the double mutants on YES plates incubated at different temperatures, as compared with that of the corresponding single mutants and the WT strain. Table 2 shows the data obtained from this analysis.

We found that the *cfh3Δ* mutant exhibited a genic interaction with *cps8-188* (carrying a point mutation in the $act1^{+}$ gene, coding for actin), *cdc15-140* (affected in a protein essential for the assembly of the ring), *cdc14-118* (affected in the SIN pathway), *cps1-19,* (a mutant in the glucan synthase required for the synthesis of the primary septum), and with *imp2Δ* (devoid of a protein from the same family as Cdc15p that seems to act in the disassembly of the ring after its

Table 2. Genetic interaction of *cfh3Δ* with cytokinesis mutants

| Mutant / Temperature (ºC) | 22 | 25 | 28 | 30 | 32 | 33 | 35 | 37 |
|---|---|---|---|---|---|---|---|---|
| *cps8-188* | | | ++ | ++ | ++ | +± | -- | |
| *cps8-188 cfh3Δ* | | | ++ | +± | -- | -- | -- | |
| *cdc15-140* | | | ++ | ++ | ++ | ± | | |
| *cdc15-140 cfh3Δ* | | | ++ | +± | ± | -- | | |
| *myo2-E1* | ++ | ++ | ++ | ++ | ++ | ++ | | |
| *myo2- E1 cfh3Δ* | ++ | ++ | ++ | ++ | ++ | ++ | | |
| *myo3Δ* | ++ | ++ | ++ | ++ | ++ | ++ | | |
| *myo3Δ cfh3Δ* | ++ | ++ | ++ | ++ | ++ | ++ | | |
| *myo2-E1 myo3Δ* | +± | +± | +± | +± | +± | ++ | | |
| *myo2- E1 myo3Δ cfh3Δ* | +± | +± | +± | +± | +± | ++ | | |
| *cdc4-8* | | | ++ | ++ | ++ | ++ | | |
| *cdc4-8 cfh3Δ* | | | ++ | ++ | ++ | ++ | | |
| *chs2Δ* | | | ++ | ++ | ++ | ++ | ++ | ++ |
| *chs2Δ cfh3Δ* | | | ++ | ++ | ++ | ++ | ++ | ++ |
| *cdc11-119* | | | | | ++ | ++ | -- | -- |
| *cdc11-199 cfh3Δ* | | | | | ++ | ++ | -- | -- |
| *cdc14-118* | | | ++ | ++ | +± | ± | -- | -- |
| *cdc14-118 cfh3Δ* | | | ++ | ++ | -- | -- | -- | -- |
| *imp2Δ* | ++ | ++ | ++ | ++ | ++ | ++ | | |
| *imp2Δ cfh3Δ* | ± | ± | +± | ± | ± | - | | |
| *cps1-191* | ++ | ++ | ++ | ++ | ++ | ++ | | |
| *cps1-191 cfh3Δ* | + | + | + | ± | -- | -- | | |
| *cfh3Δ* | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ |
| WT (HVP30) | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ |

++ Normal growth  +± Moderate growth  + Slow growth  ± weak growth  -- No growth

contraction).

In figure 23 it is shown how the double *cps8-188 cfh3Δ*, *cdc15-140 cfh3Δ*, *cdc14-118 cfh3Δ*, *cps1-191 cfh3Δ*, and *imp2Δ cfh3Δ* mutants are not able to grow at the indicated temperatures whereas each corresponding single mutant and the WT are able to grow. When the cells were streaked onto YES supplemented with 1.2 M sorbitol (an osmotic stabilizer that improves the growth of mutants affected in the synthesis of the cell wall), the *cps8-188 cfh3Δ*, *cdc15-140 cfh3Δ*, *cdc14-118 cfh3Δ* and *cps1-191 cfh3Δ* strains were able to grow in the present of sorbitol, indicating that this strain is affected in the cell wall. Surprisingly, the double *imp2Δ cfh3Δ* mutant did not grow in the presence of sorbitol (figure 23), indicating that in this double mutant the defect affects other processes that can not be suppressed adding the osmotic stabilizer. Sorbitol is also a stress-inducing agent so it is possible that this double mutant is particularly sensitive to stress.

In conclusion, all the results detailed in section 3 showed that the Cfh3p exerts its function at the time of CAR assembly and/or contraction or during septum synthesis. To get more information about the function

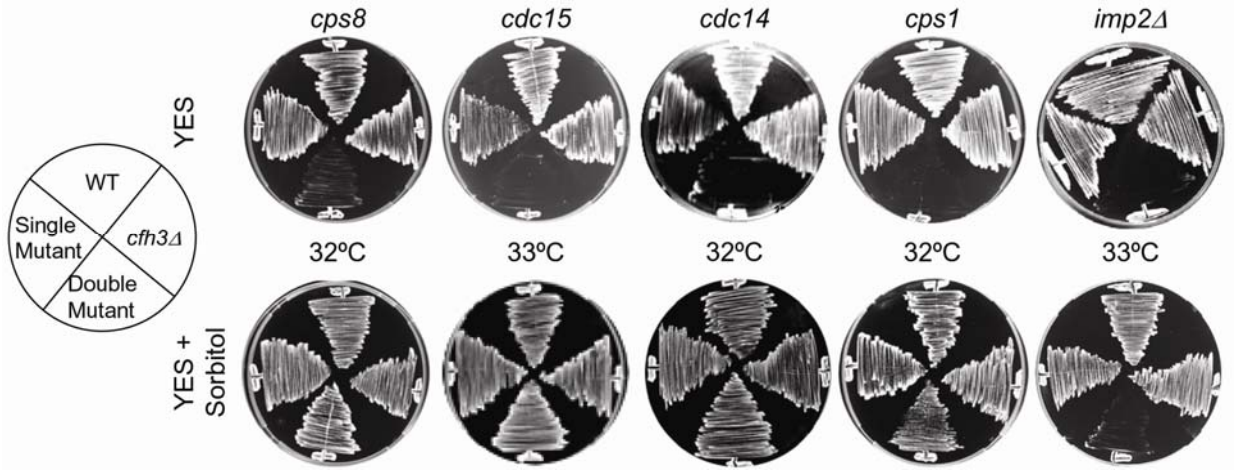



**Figure 23. The *cfh3Δ* mutant shows a genic interaction with mutants affected in cytokinesis**. The cells from the indicated mutants were streaked onto YES plates (upper panels) or YES with 1.2 M sorbitol (lower panels) and incubated at the indicated temperatures for 2 days.

of Cfh3p, we studied in more detail the relationship between Cfh3p and the component of the CAR, and the relationship between Cfh3p and proteins involved in septum synthesis.

## 4. Relationship between Cfh3p and components of the CAR

In order to get more information about the relationship between Cfh3p and some component of the CAR we performed the experiments described below.

### 4.1. Cfh3p co-localizes with actin but does not require actin to remain at the ring

First, we analyzed co-localization between GFP-Cfh3 and actin (stained with Rhodamine-Phalloidin) in fixed cells. We found that Cfh3p co-localizes with actin at the CAR but not at the patches (figure 24 A). This result was confirmed by analyzing the co-localization between RFP-Cfh3 and GFP-Crn1 (coronin, a patch-associated protein; Pelham & Chang, 2001. Figure 24 C). These result showed that Cfh3p is not associated to all the actin-containing structures. Then, we wondered whether Cfh3p remained associated to the CAR in the absence of actin. To do so, we used 100 μM of latrunculin A (a drug that depolymerizes actin) and we observed that Cfh3p stayed at the midzone at a time of treatment (15 minutes) at which actin was depolymerized (figure 24 B). These results indicated that Cfh3p co-localizes with actin at the CAR and that, once Cfh3p is at the cell midzone, its permanence there does not depend on actin and, therefore, it must remain associated to other protein(s).

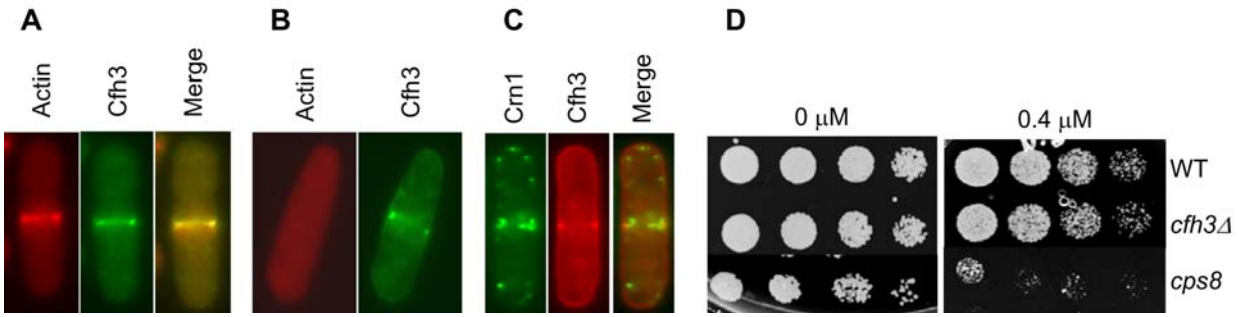



**Figure 24. Relationship between Cfh3p and actin**. (A) Cfh3p co-localizes with actin at the contractile ring. Rhodamine-Phalloidin (Actin), GFP (Cfh3), and merge fluorescence is shown. (B) Cfh3p stays at the cell midzone after actin is depolymerized with Latrunculin A. Rhodamine-Phalloidin (Actin) and GFP (Cfh3) fluorescence is shown. (C) Cfh3p does not co-localize with coronin (Crn1). GFP(Crn1), RFP (Cfh3) and merge fluorescence is shown. (D) The *cfh3Δ* mutant is not hypersensitive to actin depolymerization. $3x10^4$ cells from the indicated strains and serial 1:4 dilutions were spotted onto YES plates supplemented with the indicated concentrations of Latrunculin A and incubated at 25ºC for 3 days before being photographed.

### 4.2. Growth in the presence of Latrunculin A

To study the relationship between Cfh3p and actin more deeply, we decided to study the growth of the *cfh3Δ* mutant respect to that of the WT control in YES plates supplemented with low concentrations of latrunculin A (see materials and methods). The idea was to analyze if in the absence of Cfh3p the sensitivity to this drug increased, suggesting that Cfh3p would play a role in actin polymerization or regulation. $3x10^4$ cells and serial 1:4 dilutions from the indicated strains were spotted onto YES plates supplemented with different concentrations of the drug. As shown in figure 24 D, there was no difference in the growth of the WT and the mutant strains, suggesting that Cfh3p does not play a role in actin polymerization. As a control, the growth of a *cps8-188* strain, which carries a point mutation in the *act1*$^+$ gene, was analyzed.

### 4.3. Cfh3p co-localizes and co-immunoprecipitates with Cdc15p

As explained above, we found a relationship between Cfh3p and Cdc15p. In order to study this relationship deeply, we analyzed if Cdc15p and Cfh3p co-localized by transforming a strain bearing the GFP-fused Cdc15 protein with an integrative plasmid carrying a RFP-tagged Cfh3 protein, and observing this strain under the fluorescence microscope. As shown in Figure 25 A, Cfh3p co-localized with Cdc15p at the cell equator.

In order to know whether Cdc15p and Cfh3p have a physical interaction in the cell, we performed co-immunoprecipitation assays. We incubated cell extracts from the strains carrying the HA-tagged Cfh3p, GFP-tagged Cdc15p, or both, in the presence of rabbit anti-GFP antibody. Western blotting analyses were performed using mouse anti-GFP or anti-HA antibodies to detect the immunoprecipitation. In parallel, total cell extracts from the same strains were analyzed by Western blotting to observe the input loading. As shown in figure 25 B, HA-Cfh3p was detected in anti-GFP immunoprecipitates from the strain bearing both GFP-Cdc15p and HA-Cfh3p, but not from the control strains, pointing to a physical interaction (direct or in a complex) between both proteins. This result showed that Cfh3p is a CAR-associated protein.

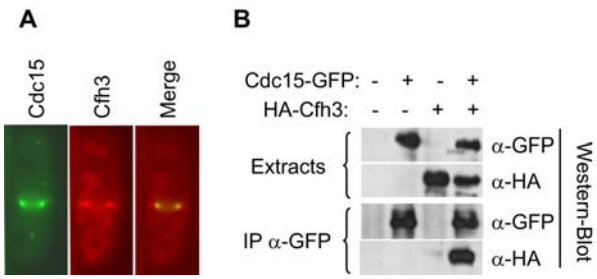



**Figure 25. Cfh3p is a ring-associated protein**. A. Cfh3p co-localizes with the ring protein Cdc15p. Left panel: GFP-tagged Cdc15p; central panel: ToRFP-tagged Cfh3p; right panel: merged images. (B) Cfh3p and Cdc15p co-immunoprecipitate. Cell extracts from strains carrying the Cdc15-GFP and/or the HA-Cfh3 fusion proteins were analyzed by Western blotting using monoclonal anti-GFP (α-GFP) or anti-HA (α-HA) antibodies before (Extracts) or after immunoprecipitation (IP) with a polyclonal anti-GFP antibody.

### 4.4. Cfh3p ensures CAR stability in cells suffering stress

#### 4.4.1. Time-lapse analysis of ring contraction

Since we had found that Cfh3p is associated to the CAR, we wanted to know whether the *cfh3Δ* cells had a defect in the ring assembly/contraction processes. To get information about how the ring behaved in the absence of Cfh3p, time-lapse analyses of ring contraction were performed. WT or *cfh3Δ* strains that carried GFP-fused Cdc15p and Hht2p (a histone) were used to visualize the progression CAR contraction and nuclear division along time. In this way, the photographs could be compared at the same time points.

Initially, sample preparation was performed by collecting the cells by centrifugation, resuspending them in a small volume of medium and mixing equal volumes of the liquid culture and melted solid medium kept at 42ºC (see materials and methods). Our initial results showed that the time for CAR assembly and contraction for the control strain was about 40 ± 3 minutes (n= 10), while this time for the *cfh3Δ* strain was about 75 ± 9 minutes (n=7). Since the *cfh3Δ* mutant does not show either a delay in the generation time or an increase in the number of septated cells, we wondered if this surprising result was a consequence of the method used to prepare the samples.

Then, the experiment was repeated using a different method to prepare the samples; cells were collected by filtration, resuspended in a small volume of

liquid medium, and spread onto solid medium that was placed into a cavity of the slide. When we used this method for sample preparation, the time required for the assembly and contraction of the ring in the *cfh3Δ* and the WT strains was 40 ± 3 minutes (n=10). This result was in agreement with the similar percentage of septated cells in the *cfh3Δ* and WT strains and suggested that in the *cfh3Δ* mutant Cdc15p rings were sensitive to stress.

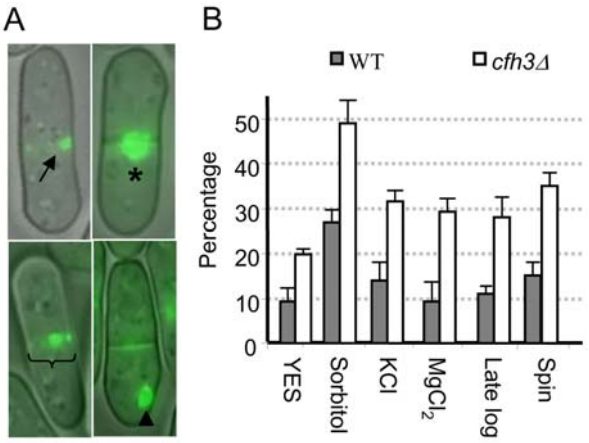



**Figure 26. Cfh3p is required for ring stability**. (A) Overlaid DIC and fluorescence images of cells collected by centrifugation. The arrow points to an asymmetric ring; the asterisk shows a ring that did not disassemble after the septum had been completely synthesized; the bracket marks a broken ring, and the arrowhead points to an abnormal accumulation of Cdc15p in the cell body. (B) Percentage of cells with an abnormal distribution of Cdc15p. The standard deviation is given for each value.

### 4.4.2. Analysis of the CAR under stress conditions

In order to confirm that the contractile rings are sensitive to stress in the *cfh3Δ* mutant, cells carrying the Cdc15-GFP protein were incubated in the presence of different sources of stress at 32ºC, collected by filtration, and observed under the fluorescence microscope. Abnormal Cdc15p distribution included asymmetric rings (50 % of the cases; see the arrow in figure 26 A), rings that did not disassemble properly (20% of cases; asterisk), broken rings (25% of the cases; bracket) or accumulation of the protein at the cytoplasm (5% of the cases; arrowhead). The total number of cells with an abnormal distribution of Cdc15p was scored. The results, shown in figure 26 B, are the media of at least 3 experiments and are detailed bellow:

**YES Medium.** A small number of cells from the WT strain exhibited an abnormal localization of Cdc15p (9%, n=500) when they were incubated in YES medium at 32ºC and collected by filtration. For the *cfh3Δ* cells this number was 19% (n=539). This result suggested that CARs are unstable in the absence of the Cfh3p even when the cells are growing under normal laboratory conditions.

**YES supplemented with sorbitol.** Sorbitol is an osmotic protector for the mutants with a defective cell wall; however, when the cells are transferred from a sorbitol-devoid medium to a sorbitol-containing medium they suffer an osmotic shock so this compound can also be considered as a stressing agent. We incubated WT and *cfh3Δ* cells in YES medium, collected them by filtration and transferred them to YES medium or to YES medium supplemented with 1.2 M sorbitol. After 15 minutes at 32ºC, the cells were collected by filtration in order to concentrate them and to prepare the slides for observation. The abnormal distribution of Cdc15p was scored. The results showed that the number of cells with abnormal rings increased in YES plus sorbitol medium with respect to YES medium, in both the WT and the *cfh3Δ* strains. This increase was from 9% to 26% (n=795) for the WT, and from 19% to 49% (n=1346) for the *cfh3Δ* strain (figure 26 B).

**YES supplemented with KCl or with $MgCl_2$.** KCl is also an osmotic-stressing agent. Cells were exposed to either 0.6 M or 1 M KCl in YES medium for 15 minutes and collected by filtration. Under this source of stress, cells showed an increase in the number of abnormal ring in both, the WT and the *cfh3Δ* strains. For the WT strain the number of cells with an abnormal Cdc15p distribution went up to 14% of the cells (n=1291), and for *cfh3Δ* this number went up to 32% (n=1762) (figure 26 B). In the presence of $MgCl_2$, 9% of the WT cells exhibited abnormal rings, while this value was 29% in the *cfh3Δ* strain.

**Nutritional stress.** In order to check whether other sources of stress had the same effect in the CAR, cells were inoculated in YES medium at an $OD_{600}$=0.4 and incubated at 32ºC for 8 hours so the cells were entering the stationary phase. Cells were collected by filtration. Under this condition, there was an increase in the number of cells from the WT and *cfh3Δ* strains that showed an abnormal distribution of Cdc15p

(figure 26 B, Late log). For the WT strain this number went up to 11% of the cells (n=543), and for the *cfh3Δ* mutant it went up to 28% (n=717).

**Mechanical stress.** As shown in the 4.4.1 section, cell showed some defect in Cdc15p distribution when the samples were prepared by centrifugation, which represents a mechanical stress. In order to study this phenomenon with more detail, cells were incubated in YES medium at 32ºC and collected by centrifugation (2 minutes at 3000 rpm). We found that this mechanical stress produced an increase in the number of cells showing an abnormal Cdc15p distribution in both, the WT and the *cfh3Δ* strains (figure 26 B, spin). For the WT, the number of cells with abnormal rings went up to 15% (n=557), and for *cfh3Δ* it went up to 35% (n=583).

In sum, all the experiments described in this section showed that stress affects the distribution of Cdc15-GFP and that in all the conditions tested the defect in the ring is significantly higher in the *cfh3Δ* strain than in the WT. All the above experiments were repeated using a GFP-fused Cdc4 protein and we got similar results. When the strains were incubated with KCl or sorbitol for longer times (2 hours), the distribution of normal/abnormal rings was similar in the cells incubated in YES or in YES supplemented with sorbitol or KCl (not shown). These results showed that the contractile rings were more unstable in the *cfh3Δ* strain than in the WT, particularly when the cells suffered some stress, and that the WT and *cfh3Δ* cells were able to recover from the initial stress shock and to repair the damages in the CARs.

## 5. Relationship between Cfh3p and glucan synthesis

Since we had found a relationship between *cfh3*$^+$ and the SIN pathway, it was also possible that Cfh3p acted at the time of septum synthesis. This was in agreement with the fact that we had found a genic interaction between *cfh3Δ* and the *cps1-191* mutant, affected in the *bgs1*$^+$/*cps1*$^+$ gene, which codes for the β-glucan synthase required for the synthesis of the primary septum. Therefore, we wanted to study the relationship between *cfh3*$^+$ and the process of cell wall/septum synthesis in more detail. To do so, we performed the experiments detailed below.

### 5.1. Sensitivity of *cfh3Δ* mutants to antifungals that inhibit β-glucan synthesis

Caspofungin is an antifungal drug, belonging to the family of echinocandins. It inhibits β(1,3)glucan synthesis thereby disturbing the integrity of the fungal cell wall and shows activity against *Aspergillus* and *Candida*. We used this drug to analyze if *cfh3Δ* mutants showed a defect in cell wall/β-glucan synthesis. $3x10^4$ cells and serial 1:4 dilutions from the WT, *cfh3 Δ*, *cps1-191* and *cps1-191 cfh3Δ* strains were spotted onto YES plates supplemented with different concentrations of Caspofungin (0.0 μg/ml, 0.75 μg/ml, 1.5 μg/ml and 2.0 μg/ml). We found that the *cfh3Δ*, *cps1-191*, and *cfh3Δ cps1-191*cells were more sensitive than the WT at 2 μg/ml of Caspofungin. The *cfh3Δ cps1-191* double mutant was the most sensitive of all the strains, since it did not grow at a concentration (1.5 μg/ml) at which the single mutants were only partially sensitive (figure 27 A).

The experiment was also performed with Enfumafungin, another echinocandin. The experiment was done using three concentrations, 3.0 μg/ml, 5.0 μg/ml and 7.0 μg/ml. It was observed that the single *cps1-191* and *cfh3Δ* mutants were hypersensitive at the 7 μg/ml of Enfumafungin (figure 27 A), and that the double *cps1-191 cfh3Δ* mutant was slightly sensitivity to 5.0 μg/ml of enfumafungin (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.

### 5.2. Sensitivity of *cfh3Δ* mutants to digestion by glucanases

This method is used for a rough measurement of the cell wall state. Glucanase sensitivity of *S. pombe* cells can be due to different causes such as a decrease in the amount of cell wall glucan or an increase in the permeability of the cell wall to the enzyme. The sensitivity to enzymatic degradation can be used to corroborate other results that suggest a defective cell wall (Perez & Ribas, 2004). The experiment was performed

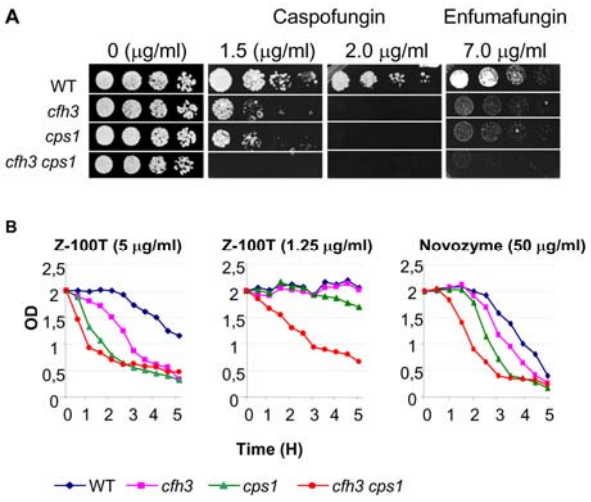



**Figure 27. The *cfh3Δ* mutant is sensitive to inhibitors of β-glucan synthesis and to β-glucanases**. (A) $3x10^4$ cells and serial 1:4 dilutions from the indicated strains were spotted onto YES plates or YES plates supplemented with the indicated amounts of Caspofungin and Enfumafungin and were incubated at 25ºC for four days. (B) Lysis of cell suspensions from the indicated strains grown in YES medium at 25ºC. Cells were incubated in the presence of the indicated concentration of Zimolyase-100T or Novozyme 234; samples were taken every 30 minutes and their OD600 nm was estimated. The experiment was performed three times, with similar results. A representative experiment is shown.

using two sources of glucanases, Zymolyase-100T and Novozyme 234. Zymolyase-100T mainly degrades β (1,3)glucan and galactomannan, and Novozyme 234 is a crude extract able to degrade completely the *S. pombe* cell wall (Perez & Ribas, 2004). The strains used were the WT and the *cps1-191*, the *cfh3Δ*, and the *cps1-191 cfh3Δ* mutants. Cells were grown in YES medium at 25ºC and incubated at 28ºC in 50 mM citrate-phosphate buffer in the presence of the β-glucanases.

As shown in figure 27 B, the lysis rates of the WT and the mutant cells grown at 25ºC were different in the presence of these enzymes. In the presence of 50 µg/ml of Novozyme 234, the *cfh3Δ* cells showed a faster lysis rate than that of the WT cells, the *cps1-191* mutant showed a faster lysis rate than that of the *cfh3Δ* mutant cells, and the double *cps1-191 cfh3Δ* mutant exhibited the fastest lysis rate respect to all the strains. Similar results were obtained with 5 µg/ml of Zymolyase-100T. The cells from the double mutant were extremely sensitivity to the degradation with this glucanase, since they lysed very fast in the presence of 1.25 µg/ml Zymolyase-100T. Thus, these results indicated that there was a defect in the integrity of the cell wall of the mutants and that the strongest defect was that of the double *cps1-191 cfh3Δ* mutant.

### 5.3. Cell wall composition of *cfh3Δ* mutants

In order to get more exact information about the cell wall composition of the *cfh3Δ* strain, we decided to quantify the amount of the different polymers in this mutant and to compare it with that of the WT strain and that of the *cps1-191* and the *cfh3Δ cps1-191* strains. Chemical and enzymatic analysis of labeled cell walls is the best method to quantify the different polymers (Perez & Ribas, 2004). We have used a procedure, described in materials and methods, to quantify the two major polymers, β(1,3)glucan and α(1,3) glucan, as well as the galactomannan from the indicated strains grown at 25ºC in the presence of [$^{14}$C] glucose for 6 hours. The experiments were performed 5 times with duplicates. The results obtained from this analysis (figure 28 A) indicated that incorporation of radioactive glucose into the cell wall was almost similar in the WT and the *cfh3Δ* mutant (43.2% and 44.9%, respectively), with differences in cell wall composition (see Figure 28). In the *cfh3Δ* mutant grown at 25ºC, there was a small but reproducible decrease in the β-glucan content respect to that of the WT strain (20% of the total [$^{14}$C] glucose compared to 22.7%) and a specific increase in the α-glucan (16.3% of the total [$^{14}$C] glucose incorporated into the cells compared to 14%) that partially compensated for the decrease in the β-glucan.

The results obtained from this analysis also indicated that incorporation of radioactive glucose into the cell wall was different in the wild type and the *cps1-191* mutant grown at 25ºC, with significant differences in the cell wall composition (Figure 28). The *cps1-191* mutant had an increase in the total cell wall incorporation of [$^{14}$C] glucose (from 43.2% to 48.6%) and exhibited a decrease in β-glucan (from 22.7% to 19% of the total [$^{14}$C] glucose incorporated) and a specific increase in the α-glucan content (from 14% to 20.9% of the total [$^{14}$C] glucose incorporated into the cells) that compensated for the decrease in β-glucan.

Respect to the double *cfh3Δ cps1-191* mutant grown at 25ºC, the results also indicated that incorporation of radioactive glucose into the cell wall in this strain was different from that of the WT strain, with

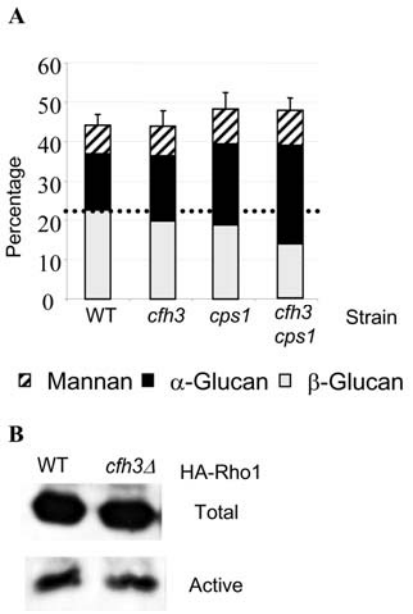



**Figure 28. The *cfh3Δ* mutants has a defect in β-glucan synthesis due to a defect in the catalytic subunit**. (A) Percentage of incorporation of radioactivity into the cell wall polysaccharides of the indicated strains. For comparison, the dotted line marks the level of β-glucan in the WT strain. (B) Total and active HA-tagged Rho1p in cell extracts from the indicated strains.

significant differences in the cell wall composition (figure 28 A). The double *cfh3Δ cps1-191* mutant had an increase in the total cell wall incorporation of the [$^{14}$C] glucose (from 43.2% to 48%). There was a significant decrease in the β-glucan content (from 22.7% to 13.7% of the total [$^{14}$C] glucose incorporated in the cells) and a specific and significant increase in the α-glucan content (from 14% to 24.8% of the total [$^{14}$C] glucose incorporated into the cells) that compensated for the decrease in β-glucan.

These results corroborated those obtained previously by analyzing sensitivity to antifungal drugs and sensitivity to digestion by glucanases, and confirmed that the *cfh3Δ*, the *cps1-191* and *cfh3Δ cps1-191* mutants have a defect in the biosynthesis of the cell wall in general, and a reduced synthesis of the β-glucan in particular.

### 5.4. Rho1 activity in *cfh3Δ* mutants (Rhotekin assay)

As shown above, Cfh3p seemed to be implicated in the biosynthesis of the β-glucan component of the cell wall. Since Rho1p is a regulator of the β-glucan synthases, we decided to check whether the defect in β-glucan synthesis in the *cfh3Δ* mutant was a consequence of a low activation of Rho1p. To analyze this possibility, we performed rhotekin binding assays to evaluate the amount of active Rho1p in the *cfh3Δ* mutant, compared to that in the WT. In this test, the amount of GTP-bound Rho1p in different strains is evaluated by allowing it to bind GST-RBD (RhoA Binding Domain from the rhotekin protein). This peptide only binds the Rho1 molecules when they are GTP-bound i.e, in its active state. In this way, by precipitating the GST-RBD peptide with glutathione-sepharose beads the cellular fraction of active Rho1p can be pulled down. In parallel, an anti-HA Western blot is performed to analyze the total Rho1p in the precipitates. The strains used in this experiment carried an HA-tagged Rho1 protein and were grown in YES medium. The results obtained from the rhotekin-binding analyses showed that the amount of active Rho1p was the same in WT and *cfh3Δ* strains and, therefore, that Cfh3p does not seem to play any role in the activation of this GTPase (figure 28 B).

## 6. Relationship between Cfh3p and Bgs1/Cps1p

The results obtained analyzing the sensitivity to antifungal drugs, the sensitivity to digestion with glucanases, and the cell wall composition of *cfh3Δ* mutants showed that the *cfh3Δ* mutant had a defect in glucan synthesis and therefore that Cfh3p was likely implicated in the synthesis of β-glucan. Additionally, the genic interaction between the *cfh3Δ* and *cps1-191* and the rhotekin-binding assay suggested that the defect in the glucan synthesis was probably due to a defect in the Bgs1/Cps1p glucan synthase. Accordingly, we decided to study the relationship between Cfh3p and Bgs1/Cps1p more deeply. To do this we preformed the experiments described below.

### 6.1. Complementation of the *cps1-191* mutant by *cfh3*$^{+}$

Since the *cps1-191* mutant is ts, we wished to know whether high copies of Cfh3p were able to rescue its thermosensitivity, a result that would indicate that Cfh3p might be able to somehow activate or stabilize the Bgs1 protein in the *cps1-191* mutant. Accord-

ingly, the *cps1-191* mutant was transformed with the pAU (empty multicopy vector) or pAU*cfh3*$^+$ plasmids. The transformants were streaked onto MM-ura plates that were incubated at different temperatures (28ºC, 30ºC, 32ºC, 34ºC, 35ºC, and 37ºC). At 32ºC, the *cps1-191* cells carrying the pAU*cfh3*$^+$ plasmid grew better than the cells carrying the empty vector. At higher temperatures neither of them grew (figure 29 A and results not shown). In conclusion, these results showed that a high amount of Cfh3p was able to partially complement the ts phenotype of *cps1-191* mutant.

### 6.2. Cell morphology

Germination of *bgs1Δ* spores results in slow-growing, large, elongated, branched, and multiseptated cell units that eventually die. In *bgs1*$^+$ shut-off cells in the presence of sorbitol, cells are able to survive for at least 3 days, although cells are elongated, multiseptated and lose polarity making branches in the poles (Cortes *et al.*, 2007). Having the above information into account, we wanted to study the morphology of the *cfh3Δ* and the *cfh3Δ cps1-191*mutants respect to the WT and the *cps1-191* strains in the presence and the absence of sorbitol.

We found that the WT and the *cfh3Δ* cells did not show any apparent difference in their cell shape and viability when these strains were incubated in YES or YES medium supplemented with 1.2 M sorbitol at 32ºC. Incubating *cps1-191* mutant cells in YES medium at 32ºC produced some round, swollen, and in some cases, dead cells. In the presence of sorbitol at 32ºC, *cps1-191* cells were viable and some cells showed 2 septa (figure 29 B). In YES medium, the double *cfh3Δ cps1-191* mutant showed swollen, elongated, branched, multiseptated cells that lose polarity making branches in the poles reminding the phenotype of the *bgs1*$^+$ shut-off cells, and dead cells. Incubating the double *cfh3Δ cps1-191* cells in the presence of sorbitol did not alleviate this phenotype (figure 29 B). Furthermore, the double *cfh3Δ cps1-191* mutant showed a phenotype similar to that of the *bgs1*$^+$ shut-off cells even when they were incubated in YES medium at 25ºC (not shown). These results are in agreement of Cfh3p and Bgs1p being functionally related.

### 6.3. Calcofluor staining

It has been shown that in *S. pombe* Calcofluor white binds specifically the linear β(1,3)glucan, which is synthesized by Bgs1p (Cortes *et al.*, 2007). In order to find out whether the *cfh3Δ* mutants had any defect on the synthesis of linear β(1,3)glucan, the cells were stained with different concentrations of this dye. We found that at 0.125 µg/ml, Calcofluor stained all the septa in the WT cells (figure 29 C) but, under the same conditions, staining was extremely weak in the septa from the *cfh3Δ* cells, although the septa were distinguishable by phase contrast microscopy. Using a slightly higher concentration (0.185 µg/ml), the septa

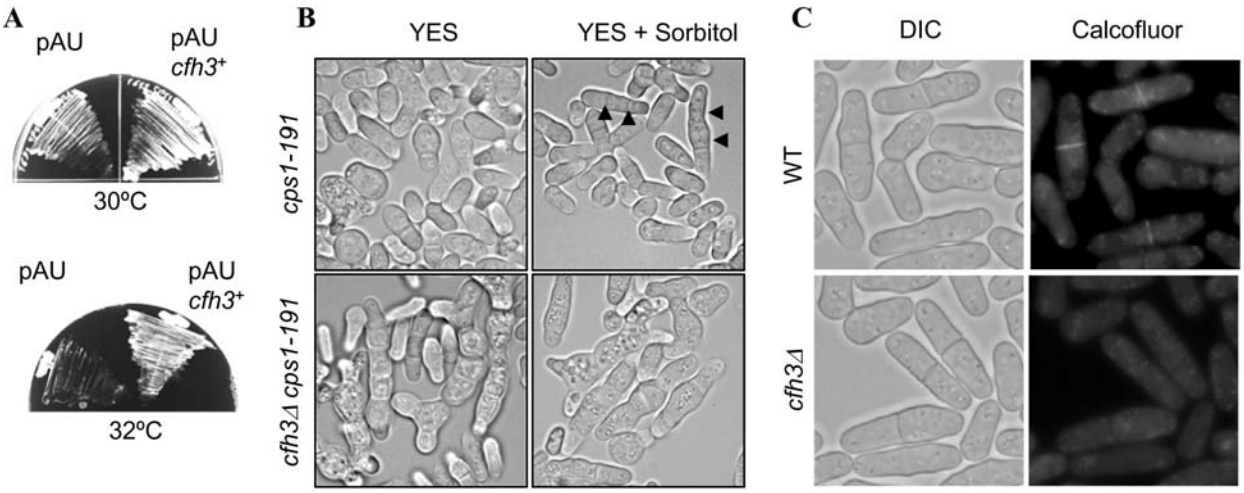



**Figure 29. Cfh3p and Bgs1p are functionally related**.(A) The cells from the *cps1-191* strain carrying either the pAU vector alone or bearing the *cfh3*$^+$ gene were streaked onto minimal medium without uracil and incubated at the indicated temperatures for three days. (B) The cells from the indicated strains were cultured in YES or YES supplemented with 1.2 M sorbitol at 32ºC and photographed. (C) DIC and fluorescence micrographs of cells from the WT or the *cfh3Δ* mutants stained with 0.125 µg/ml Calcofluor.

were stained in the *cps1-191* mutant, whereas they were not in the double *cfh3Δ cps1-191* mutant (not shown). To sum up, the results obtained from these experiments indicated that the Bgs1p activity is compromised in the *cfh3Δ* cells and supported the idea of Cfh3p having a functional relationship with Bgs1p.

### 6.4. Co-localization between Bgs1p and Cfh3p

Since Cfh3p and Bgs1p seemed to be functionally-related, we wished to know whether these proteins co-localized. Therefore, we transformed cells bearing the GFP-fused Bgs1p, with a plasmid carrying a RFP-tagged Cfh3 protein. The results showed that Cfh3p co-localized with Bgs1p in the cell equator and poles (figure 30 A), suggesting that both proteins are in close proximity.

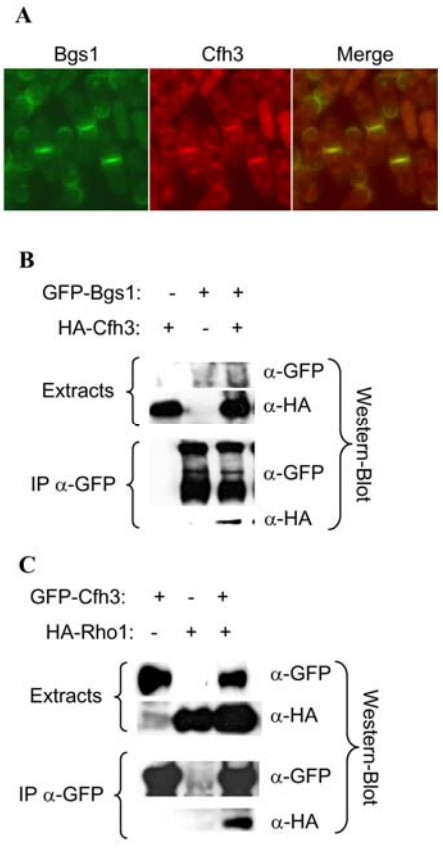



**Figure 30. Cfh3p forms a complex with the β-glucan synthase system**. (A) Bgs1p and Cfh3p co-localize. The fluorescence corresponding to GFP-Bgs1, RFP-Cfh3, and the merged images are shown. (B) Cfh3p and Bgs1p co-immunoprecipitate. Cell extracts from strains carrying GFP-Bgs1 and/or HA-Cfh3 were analyzed by Western blotting using anti-GFP (α-GFP) or anti-HA (α-HA) antibodies before (Extracts) or after (IP) immunoprecipitation with an anti-GFP antibody. (C) Cfh3p and Rho1p co-immunoprecipitate. Same as in B but the cells carried GFP-Cfh3, HA-Rho1p or both proteins.

### 6.5. Co-immunoprecipitation of Bgs1p with Cfh3p

We analyzed whether there was a physical interaction between Cfh3p and Bgs1p by performing co-immunoprecipitation assays using strains bearing an HA-tagged *cfh3*$^+$ gene, a GFP-tagged *bgs1*$^+$ gene, or both. We incubated cell extracts from these strains in the presence of a rabbit anti-GFP antibody. Western blotting analyses were performed using monoclonal anti-GFP or anti-HA antibodies to detect the immunoprecipitates. In parallel, total cell extracts from the same strains were analyzed by Western blotting in order to evaluate the input loading (see materials and methods). As seen in figure 30 B, HA-Cfh3p was detected in anti-GFP immunoprecipitates from the strain bearing both the GFP-Bgs1p and the HA-Cfh3p tagged proteins, but not from the control strains, pointing to a physical interaction between Cfh3p and Bgs1p.

### 6.6. Co-immunoprecipitation of Cfh3p with Rho1 and Cdc42

Since Bgs1p is a transmembrane protein, it is difficult to affirm that co-immunoprecipitation involving this protein means a physical interaction, since both proteins could be trapped in some micelle produced during the process of protein extraction. In order to get more information about the existence of a physical interaction between Cfh3p and the glucan synthase complex, we performed co-immunoprecipitation assays between Cfh3p and Rho1p, a functional regulator of the glucan synthases. We used strains bearing a GFP-tagged *cfh3*$^+$ gene, an HA-tagged *rho1*$^+$ gene, or both. In parallel, coimmunoprecipitation assays were performed using an HA-tagged *cdc42*$^+$ gene. Cdc42p is another Rho-type GTPase which has not been related with the β-glucan synthesis. As seen in figure 30 C, HA-Rho1p was detected in the anti-GFP immunoprecipitates from the strain bearing both HA-Rho1p and GFP-Cfh3p, but not from the control strains. On the contrary, Cdc42p was not detected in the immunoprecipitates (not shown). These results strongly suggest that there is a physical interaction between Cfh3p and the glucan synthase complex.

## 7. Relationship between Cfh3p, Bgs1/Cps1p and the CAR

As described above, we had found that the *cfh3*$^{+}$ gene played a role in maintaining CAR stability and a role in regulating Bgs1p and glucan synthesis. We wondered if these functions of the protein were related to each other or they were independent. To analyze this relationship, we studied if Bgs1p played a role in CAR stability by observing how the contractile ring behaves in the *bgs1-191* mutant incubated under stress conditions. We observed the Cdc15-GFP rings in the strains of interest that had been pre-incubated in YES medium at 25ºC (permissive temperature for the *cps1-191* mutation) and then had been incubated for 15 minutes either in the same medium at 25ºC or at 32ºC, or in YES plus 1.2 M sorbitol (osmotic shock) at 32ºC. We quantified the cells with normal or abnormal distribution of Cdc15-GFP as explained in the section 4.4.2. We found that in all the conditions the *cps1-191* mutant exhibited more cells with abnormal rings than the *cfh3Δ* mutant and that the *cfh3Δ cps1-191* double mutant showed the strongest defect (figure 31). Thus, 35% of the *cps1-191* cells exhibited abnormal Cdc15 rings at the permissive temperature and that this defect was observed in 62% of the cells incubated at 32ºC and in up to 83% of the cells (n= 500 in all cases) when the culture had suffered the osmotic shock. The percentages for the *cfh3Δ cps1-191* strain were 42%, 76%, and 91% (n= 500 in all cases) for the YES cultures incubated at 25ºC or at 32ºC, and for the YES plus sorbitol culture incubated at 32ºC, respectively. The results shown in figure 31 are the media of at least 3 experiments. In conclusion, these results showed that there is a defect in the Cdc15p distribution in the *cps1-191*mutant even in normal growth conditions, pointing to CAR instability in this mutant. Under osmotic stress, this defect was further enhanced in the *cps1-191* mutants, showing that stress affected the Cdc15p distribution when the Bgs1/Cps1 glucan synthase is not completely functional. Thus, it is possible that at least part of the instability in the Cdc15 ring observed in the *cfh3Δ* mutant is a consequence of the defect in glucan synthesis observed in this strain (see above).

## 8. Relationship between Cfh3p, Bgs1p and stress

The results described above show that in the *cfh3Δ* and the *cps1-191* mutants contractile rings are sensitive to stress. Therefore, we wondered whether in these mutants the cells were sensitive to stress and which would be the reason for this sensitivity.

### 8.1. Growth on plates supplemented with stress-inducing compounds

In order to determine the viability of *cfh3Δ* cells under stress conditions, a set of experiments were performed using the WT strain and the *cfh3Δ* mutant. As a control we used a *spm1Δ* strain, which is devoid of a MAP kinase involved in cell wall construction, cytokinesis and stress response (Toda *et al.*, 1996, Zaitsevskaya-Carter & Cooper, 1997, Madrid *et al.*, 2006). In each case, 3x10$^{4}$ cells from logarithmic cultures and 1:4 serial dilutions were spotted onto YES plates supplemented with different concentrations of

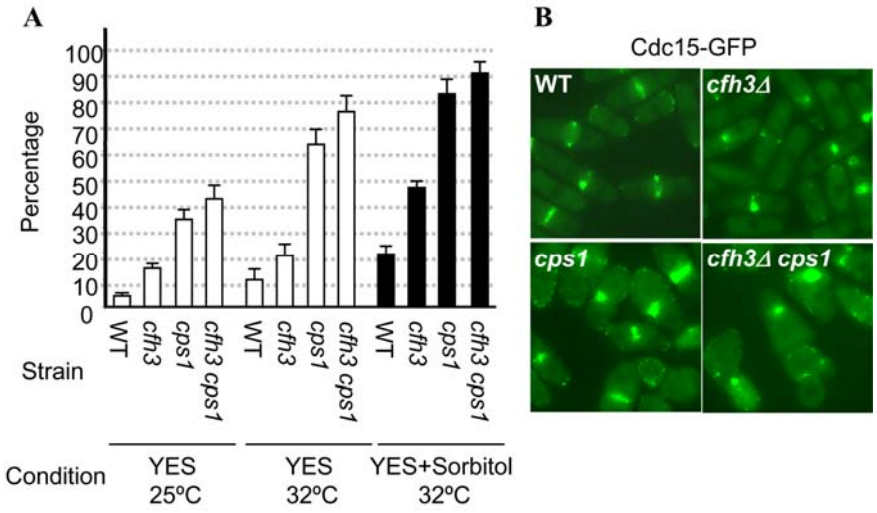



**Figure 31. Bgs1p is required for CAR stability**. (A) Percentage of cells with an abnormal distribution of Cdc15p in the indicated strains incubated at the indicated temperatures in the presence or absence of sorbitol for 15 minutes. (B) Representative fields of cultures from the indicated strains incubated in the presence of sorbitol at 32ºC for 15 minutes.

$MgCl_2$, KCl, NaCl, $H_2O_2$, and Caffeine (see materials and methods). Plates were incubated at 32ºC for 2-3 days and photographed. *cfh3Δ* and *spm1Δ* cells were slightly hypertolerant to 0.15 M NaCl and hypersensitive to 1.2 M KCl, and 6 mM caffeine, the *cfh3Δ* cells were hypersensitive to 0.1 M $MgCl_2$, and there was no difference in the growth of the WT strain and the *cfh3 Δ* mutant in the presence of hydrogen peroxide (figure 32 A and results not shown).

Then, we wanted to analyze if the *cps1-191* and the double *cfh3Δ cps1-191* mutants were also sensitive to stress. To perform this experiment, the WT strain and the *cfh3Δ*, *cps1-191*, and *cfh3Δ cps1-191* mutants were spotted onto plates of YES medium supplemented with different concentrations of Caffeine, $MgCl_2$, and KCl as explained above. We also included a strain which carries a mutation in *cwg1*$^+$*/bgs4*$^+$, another *S. pombe* glucan synthase homologue (Cortes *et al.*, 2005), and the *spm1Δ* mutant as controls. The plates were incubated for 5-7 days at 25 °C before being photographed. *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 32 B). The *cwg1-1* cells behaved as the WT did. Although being a point mutation we cannot rule out a general sensitivity of glucan-synthase mutants to stress, it seems that this might well be a characteristic of the *bgs1* mutants.

It has been suggested that Cfh3p is a regulatory protein for the chitin synthase-like protein Chs2p (Matsuo *et al.*, 2004) so we wanted to study the relationship between Chs2p, Cfh3p, and stress. Sensitivity of the WT, *chs2Δ* , *cfh3Δ*, and *chs2Δ cfh3Δ* strains to different concentrations of $MgCl_2$ was analyzed. The plates were incubated for 3 days at 32 °C before being photographed. The *chs2Δ* mutant did not show any sensitivity to $MgCl_2$ and the double *chs2Δ cfh3Δ* mutant did not show more sensitivity to $MgCl_2$ than the single *cfh3Δ* did (figure 32 C and results not shown). In conclusion, the results obtained from this experiment suggest that Cfh3p is not a regulatory protein for Chs2p.

Finally, there are three more *cfh*$^+$ genes in *S. pombe: cfh1*$^+$, *cfh2*$^+$, and *cfh4*$^+$. We wished to know whether they collaborate with Cfh3p in the regulation of Bgs1p. Then, the sensitivity of the WT, *cfh3Δ* , *cfh1 Δ cfh3Δ*, *cfh2Δ cfh3Δ*, *cfh3Δ cfh4Δ*, *cfh1Δ cfh2Δ cfh3 Δ*, *cfh2Δ cfh3Δ cfh4Δ*, and *cfh1Δ cfh2Δ cfh3Δ cfh4Δ* strains to YES medium supplemented with different concentrations of $MgCl_2$ was analyzed. The plates were incubated for 3 days at 32 °C before being photo-

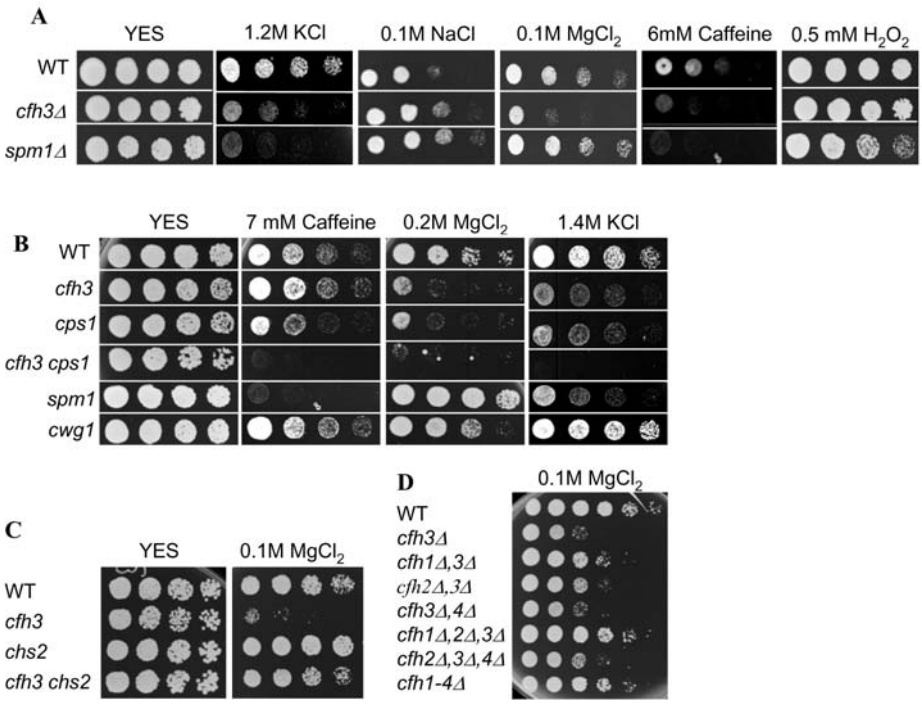



**Figure 32. Analysis of sensitivity to stress**. 3x$10^4$ cells and 1:4 serial dilutions from the indicated strains were spotted onto YES plates or YES plates supplemented with the indicated compounds and incubated at 32ºC (A, C and D) or at 25ºC (B).

graphed. The multiple *cfh* mutants did not show an enhanced sensitivity to $MgCl_2$ than single the *cfh3Δ* mutant did (figure 32 D and results not shown), indicating that the other *cfh*$^+$ genes do not collaborate with each other with respect to stress response and suggesting that the regulation of Bgs1p is specific for Cfh3p.

### 8.2. Growth of cells from stationary-phase cultures

As described above, the nutritional stress affects CARs in the *cfh3Δ* mutants so that in these mutants there was a significant increase in the number of cells with defective contractile rings. Therefore, we wanted to know whether there was any growth defect in the cells that came from the stationary phase of culture. To study this, the WT, *cfh3Δ*, *cps1-191*, and *cfh3 Δ cps1-191* strains were grown at 25ºC for 4 days in liquid YES medium. After this time, the cultures were OD-equaled and serial dilutions were spotted onto YES plates that were incubated for 3 days at 25 °C before being photographed. As shown in figure 33, the *cfh3Δ* and the *cps1-191* strains grew as the WT strain did. We found that the double *cfh3Δ cps1-191* mutant hardly grew, suggesting that this strain had a defect in growth after suffering a strong nutrient limitation. This result was in agreement with the fact that when we observed cells from this strain that had been growing in YES plates at 25ºC for more than 4 days most of the cells looked swollen and dead. For this reason, this strain had to be kept alive by reinoculating it on fresh YES plates every 2 days.

In parallel, the experiment was performed growing the above-indicated strains in liquid YES medium for a longer time (7 days) at 25ºC in order to know if the single mutants were also sensitive to this stress. The rest of the experiment was performed as explained above. As shown in figure 33, the *cfh3Δ* strain grew as the WT strain did, but the *cps1-191* and the double *cfh3Δ cps1-191* mutants showed hypersensitivity to this stress, suggesting that single the *cps1-191* strain had also a problem in exiting the stationary phase.

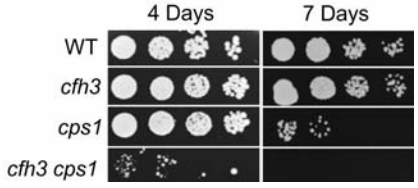



**Figure 33. The *cps1-191* and *cfh3Δ cps1-191* are sensitive to nutritional stress.** Cultures from the indicated strains were kept in liquid YES medium at 25ºC for the indicated times. Then, they were OD-equaled, $3x10^4$ cells and 1:4 serial dilutions were spotted onto YES plates and they were incubated at 25ºC.

### 8.3. Morphology of the cells grown under stress conditions

As described above, the *cfh3Δ* and *cps1-191* mutants showed abnormal contractile rings and impaired growth in the presence of some stress conditions. We wondered whether this growth defect was due to a defect in cytokinesis, as it has been described for the mutants in the Spm1p pathway (Zaitsevskaya-Carter & Cooper, 1997). These mutants exhibit cells growing as short trains joined end to end occasionally branched, when cells are exposed to some stress conditions. To uncover this, we studied the morphology of the WT, *cfh3Δ*, *cps1-191*, *cfh3Δ cps1-191*, *spm1Δ*, and *cfh3Δ spm1Δ* strains grown on solid or in liquid YES medium, or in the same media supplemented with 1.2 M sorbitol or 1.0 M KCl, and incubated at 25ºC or at 32ºC for different times. We found that, according to Hoechst staining, for each strain the morphology of the cells grown under stress conditions was similar to that shown when the cells were grown under standard laboratory conditions (figure 34 and results not shown). The morphology of the *cfh3Δ spm1Δ* double mutant was similar to that of the single *spm1Δ* mutant

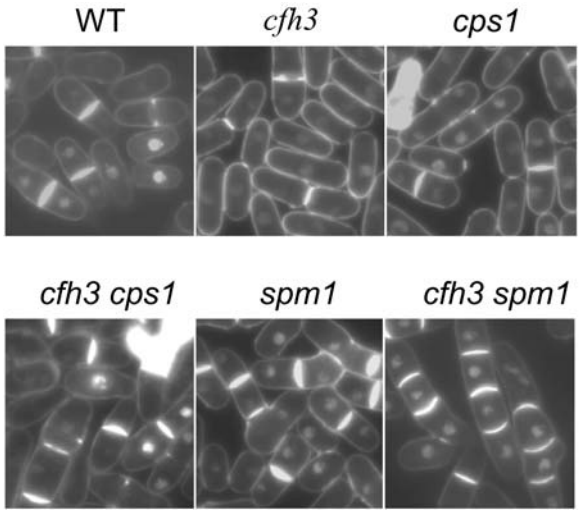



**Figure 34. Analysis of cell morphology of under stress conditions.** Morphology of the cells from the indicated strains, incubated in YES medium supplemented with 1 M KCl for 6 hours at 25ºC, and stained with Hoechst 33258.

in all conditions tested. These results showed that the sensitivity of the *cfh3Δ* and the *cps1-191* cells to stress was not due to a defect in cytokinesis and are in agreement with the fact that the cells were able to adapt to the new environment and to repair the damages produced in the CAR by the initial osmotic shock (see above).

### 8.4. Analysis of the cell wall in cells grown under stress conditions

As explained above, we have shown that the *cfh3Δ* and the *cps1-191*cells had an impaired growth in the presence of stress, although this defect was not due to an abnormal cytokinesis. In addition, we had found that these strains had a defect in glucan synthesis; therefore, we wondered whether these strains had a stronger defect in glucan synthesis under stress conditions, being this defect the reason for their impaired growth. To uncover this, we performed the experiments described in the next sections.

#### 8.4.1. Sensitivity to glucanases

This analysis was performed using two glucanase preparations, Zymolyase-100T and Novozyme 234. As explained above, Zymolyase-100T mainly degrades β(1,3)glucan and galactomannan, and Novozyme 234 is a crude extract able to degrade completely the *S. pombe* cell wall (Perez & Ribas, 2004). The sensitivity to enzymatic degradation of the WT, *cps1-191*, *cfh3Δ*, and double *cps1-191 cfh3Δ* strains was analyzed in cells that had been growing under different stress conditions (1.2 M sorbitol, 0.6 M KCl, and 0.125 M $MgCl_2$) at 25ºC for 6 hours. To perform this experiment, Novozyme was used at final concentration of 50 µg/ml and Zymolyase-100T was used at a final concentration of 5 µg/ml.

All the strains lysed significantly faster in the presence of Novozyme or Zymolyase-100T when the cells were incubated under the above-mentioned stress conditions than when they were incubated in YES medium (figure 35 A and results not shown). In all the conditions the *cfh3Δ* mutant cells lysed faster than the WT cells, the *cps1-191* cells lysed faster than the *cfh3 Δ* cells and the *cfh3Δ cps1-191* cells were the most sensitive to digestion by glucanases (not shown). This result suggested that the cell wall composition of the cells grown under stress conditions was different from that of the cells grown in YES medium, and that under stress conditions the mutants have a defective cell wall, probably due to a defect in β-glucan synthesis.

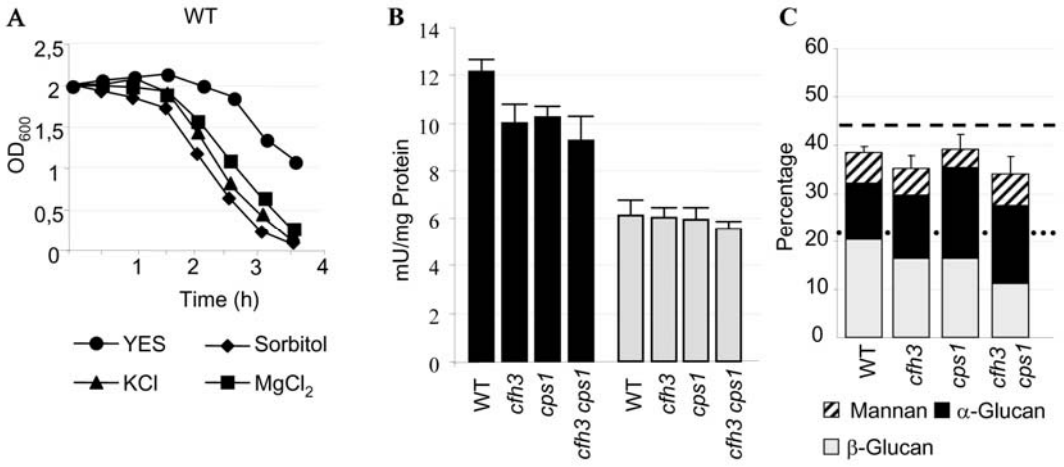



**Figure 35. Cell wall synthesis is defective in the *cfh3Δ*, *cps1-191* and *cfh3Δ cps1-191* strains grown under stress conditions.** (A) Lysis of cell suspensions from the WT strain grown in YES medium or YES medium supplemented with 1.2 M sorbitol, 0.6 M KCl, or 0.125 M MgCl2 at 25ºC for 6 hours. Cells were incubated in the presence of 50 µg/ml of Novozyme 234 for the indicated times and the OD600 nm was estimated. A representative experiment is shown. (B) β(1,3)glucan synthase activity in cell extracts from cells grown in YES (black-filled bar) or YES supplemented with 1 M KCl (grey-filled bars) for 20 minutes at 25ºC. (C) 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 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.

### 8.4.2. β-glucan synthase activity

β(1,3)glucan synthase activity was determined in the WT, *cfh3Δ* , *cps1-19*, and *cfh3Δ cps1-191*strains grown at the permissive temperature (25°C), in both standard (YES medium) and stress conditions (YES medium supplemented with 0.6 M KCl) for 20 minutes. In all cases, GTP-activated activities were assayed. As seen in figure 35 B, the specific activity was reduced to 84% and 85% in the *cfh3Δ* and the *cps1-191* strains, respectively, and to 71% in the double mutant (figure 35 B). 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. As we had expected, when the cells were grown under stress conditions, the membranous extracts from all the above-mentioned strains showed a noticeable decrease in the β(1,3) glucan synthase activity, compared to the activity detected in the membranes obtained from the cells that had been cultured under standard laboratory conditions. 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. 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.

### 8.4.3. Cell wall composition

We quantified the proportion between different polymers in the cell wall of the strains of interest incubated under stress condition by analyzing the incorporation of [$^{14}$C]glucose in the two major polymers, β(1, 3)glucan and α(1,3)glucan, as well as in the galactomannan. The WT strain and the *cfh3Δ*, *cps1-191*, and *cfh3Δ cps1-191* mutants were labeled with the [$^{14}$C] glucose in the presence of 0.6 M KCl for 6 hours at 25ºC. The results obtained from this analysis indicated that incorporation of radioactive glucose into the cell wall was significantly decreased in all the strains, with differences in the cell wall composition (figure 35 C). There was a significant decrease in β-glucan for all strains, in particular for the double *cps1-19 cfh3Δ* mutant that had about 50% of the level of this polymer present in the WT strain incubated in YES medium at 25ºC (see the dotted line in figure 35 C). Probably, this low β-glucan content contributes to the impaired growth of the mutants under stress conditions.

## 9. Co-immunoprecipitation of the β-glucan complex with Cfh3p under stress conditions

Since all the above results strongly support the idea of Cfh3p playing a role in β-glucan synthesis, which is more critical when the cells are undergoing stress, the physical interaction between Cfh3p and Bgs1p or Rho1p was analyzed in cells that had been treated with 1 M KCl for 15 minutes. Thus, co-immunoprecipitation assays were performed in strains bearing the HA-tagged *cfh3*$^{+}$ gene, the GFP-tagged *bgs1*$^{+}$, or both, and in strains bearing the GFP-tagged *cfh3*$^{+}$ gene, the HA-tagged *rho1*$^{+}$ gene, or both (figure 36). The experiments were preformed as previously described for the Cfh3p/Bgs1p or Cfh3p/Rho1p co-immunoprecipitation in cell extracts from cells that

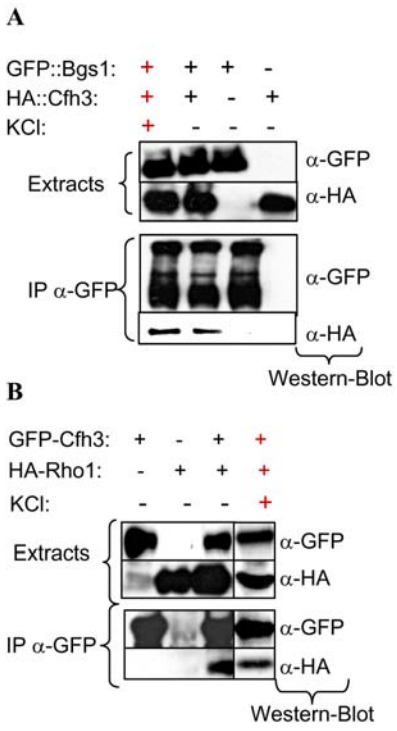



**Figure 36. Interaction between Cfh3p and the β-glucan synthase system is not disrupted by stress**. Cells bearing the indicated tagged proteins were cultured in the presence or the absence of 1M KCl for 15 minutes at 32ºC. Cell extracts were analyzed by Western blotting using anti-GFP or anti-HA antibodies before (Extracts) or after (IP) immunoprecipitation with an anti-GFP antibody.

had been incubated under normal laboratory conditions (YES medium). The results obtained from these experiments indicated that Cfh3p is also in a complex with Bgs1p and with Rho1p in when the cells are distressed (figure 36), suggesting a strong interaction between Cfh3p and the β-glucan synthase complex.

## 10. Analysis of Bgs1p regulation by Cfh3p under stress conditions

All the results described above pointed to a role of Cfh3p in the regulation of β-glucan synthesis, probably exerted through Bgs1p. However, the nature of this regulation was not clear. The experiments described below were carried out to elucidate whether Cfh3p affects the level, the posttranslational modification, or the localization of Bgs1p.

### 10.1. Analysis of the level of Bgs1p by Western blotting

With a view to understanding whether the amount or the mobility of Bgs1p was altered in the cells in the absence of Cfh3p, Western analyses were performed in cell extracts from the WT and *cfh3Δ* strains incubated in YES medium or YES medium supplemented with 1 M KCl for 15 minutes at 32ºC. The strains used carried the HA-tagged *bgs1*$^+$ gene and the GFP-tagged *cfr1*$^+$ gene (a gene that codes for a Golgi protein that was used as a loading control; Cartagena-Lirola *et al.*, 2006). As shown in figure 37 A, there was no obvious difference in the amount or in the mobility of Bgs1p in the WT and the *cfh3Δ* strains in both, standard and stress conditions.

### 10.2. Localization of Bgs1p in the *cfh3Δ* mutant

With the purpose of knowing whether Cfh3p played a role in regulating Bgs1p localization in the cells, we observed the WT and the *cfh3Δ* strains carrying a GFP-tagged *bgs1*$^+$ gene under the fluorescence microscope. Localization of Bgs1p was similar in both strains when they were incubated in YES medium (figure 37 B), although the signal in the *cfh3Δ* cells seemed to fade faster. However, when the cells were incubated for 15 minutes in the presence of 1 M KCl, Bgs1p localized properly in the WT cells (although in some cells the septal area seemed to be deformed and the signal spread along the new cell pole; see arrows in figure 37 B) but in about 80% of the *cfh3Δ* cells the protein could not be observed either at the midzone or

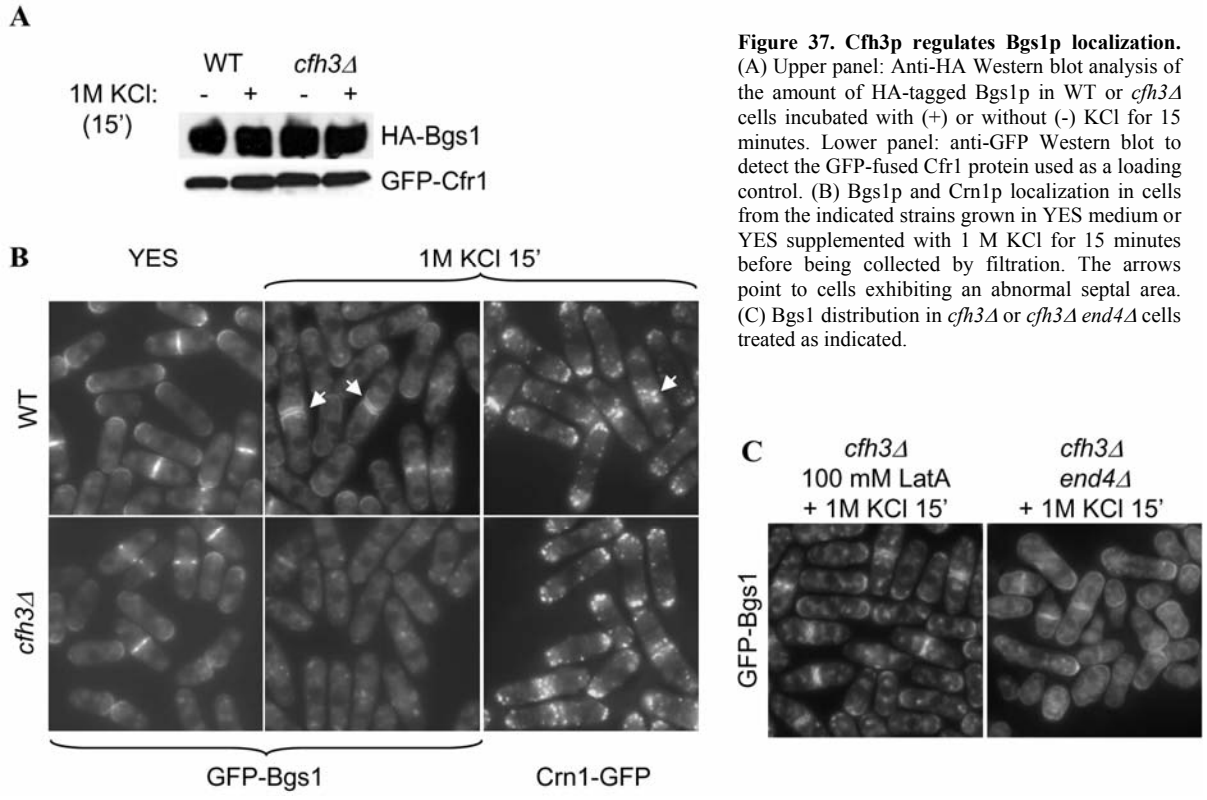



**Figure 37. Cfh3p regulates Bgs1p localization.** (A) Upper panel: Anti-HA Western blot analysis of the amount of HA-tagged Bgs1p in WT or *cfh3Δ* cells incubated with (+) or without (-) KCl for 15 minutes. Lower panel: anti-GFP Western blot to detect the GFP-fused Cfr1 protein used as a loading control. (B) Bgs1p and Crn1p localization in cells from the indicated strains grown in YES medium or YES supplemented with 1 M KCl for 15 minutes before being collected by filtration. The arrows point to cells exhibiting an abnormal septal area. (C) Bgs1 distribution in *cfh3Δ* or *cfh3Δ end4Δ* cells treated as indicated.

at the poles. The same result was obtained when the osmotic shock was produced by exposing the cells to 1.2 M sorbitol for 15 minutes (not shown). These results indicated that somehow Cfh3p regulates the localization of Bgs1p and that this function is more necessary when the cells are under stress conditions.

It is known that stress can induce actin depolarization. In order to determine if the 15 minutes incubation in the presence of 1 M KCl resulted in a different behavior of actin in the WT or the mutant strains, we performed the same experiment using WT and *cfh3 Δ* strains that carried a GFP-tagged coronin (Crn1p, a protein that associates with actin patches; Pelham & Chang, 2001). As observed in figure 37 B, this treatment did not result in a different distribution of the actin patches in the WT or the mutant strain. Then, we wanted to determine whether Bgs1p not being able to localize was because this protein was not delivered to the cell surface or because it was endocyted faster in the absence of Cfh3p and the presence of KCl. Latrunculin A is a drug that depolymerizes actin, therefore producing a blockade in endocytosis. When we treated the cells simultaneously with 100 µM latrunculin A and KCl for 15 minutes, Bgs1p could be observed at the cell surface, suggesting that Bgs1p was being endocyted in the absence of Cfh3p and the presence of KCl (figure 37 C). In any case, since latrunculin A depolymerizes actin, it was not completely clear whether proper localization of Bgs1p in the cells treated with latrunculin A was because of a blockade in endocytosis or because an alteration in the localization of Bgs1p due to the actin depolymerization. With the aim of distinguishing between these possibilities, a similar experiment was performed using *end4Δ* or *cfh3 Δ end4Δ* strains carrying the GFP-Bgs1 protein. The *end4Δ* mutant is defective in endocytosis because of the absence of a protein required at post-internalization step of this process (Iwaki *et al.*, 2004, Castagnetti *et al.*, 2005). Bgs1p properly localized in both, the *end4Δ* and the *cfh3Δ end4Δ* strains in the presence of 1 M KCl (figure 37C), strongly suggesting that Cfh3p regulates the localization of Bgs1p by regulating its endocytosis, and that this function is more necessary when the cells are under stress conditions.

In *S. pombe*, there are four glucan synthase homologues; therefore, we wondered whether Cfh3p played the same role in regulating other β-glucan synthases as it plays for Bgs1p. To answer to this question, we observed the localization of GFP-Bgs4p in the WT or *cfh3Δ* strains incubated in YES or YES supplemented with 1 M KCl and incubated for 15 minutes at 32ºC. We found that Bgs4p delocalized in both, the WT and the *cfh3Δ* strains, after the stress shock indicating that Cfh3p regulation is specific for Bgs1p (figure 38 A).

In addition, we wanted to know whether Cfh3p also regulated the localization of other membrane proteins apart from Bgs1p. In particular, we were interested in studying whether the localization of Cfhs2p (a membrane protein that has been suggested to be regulated by Cfh3p; Matsuo *et al.*, 2004) was regulated by Cfh3p under stress conditions. Therefore, a similar experiment was performed using WT and *cfh3Δ* strains carrying a GFP-tagged Chs2p. We found that Chs2p localized properly in both strains, showing that Cfh3p is not a Chs2p regulator (figure 38 B).

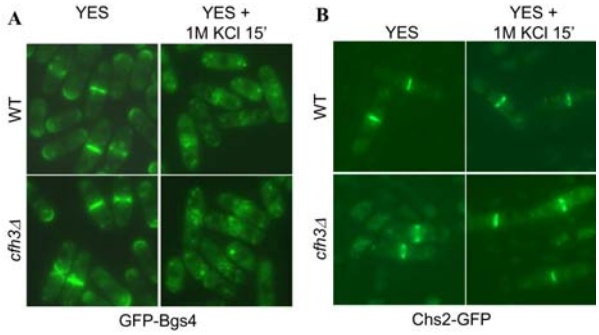



**Figure 38. Cfh3p does not regulate the localization of Bgs4p or Chs2p under stress conditions.** Cells from the WT or the *cfh3Δ* mutant carrying GFP-Bgs4 (A) or Chs2-GFP (B) were incubated in YES (left panels) or in YES with 1 M KCl (right panels) for 15 minutes before being collected by filtration.

### 10.3. Localization of Cfh3p under stress conditions

Considering above results, we wished to know how Cfh3p behaved under stress condition. Therefore, the localization of Cfh3p was studied in a WT strain incubated in YES medium or in YES supplemented with 1 M KCl for 15 minutes at 32ºC. As shown in the figure 39, Cfh3p localized properly in the medial zone and the cell poles in both media, although after the stress shock some Cfh3 protein was dispersed along the membrane, as it had been observed for Bgs1p in the WT strain treated under the same conditions (see above). The amount of Cfh3p did not vary in the cells

after the osmotic shock (Matsuo *et al.*, 2004 and our unpublished observations).

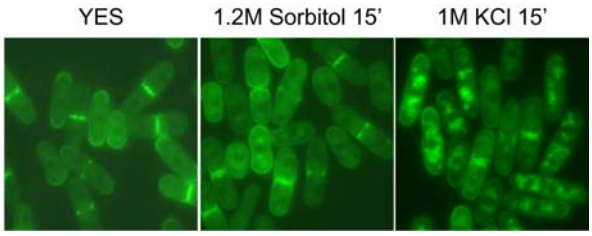



**Figure 39. Cfh3p localization under stress conditions.** Cells carrying the GFP-Cfh3p fusion protein were incubated in YES medium or YES medium supplemented with the indicated compounds at 32ºC for 15 minutes.

## 11. Relationship between *cfh3*$^+$ and the cell integrity MAP kinase pathway

The fact that the *cfh3Δ* and *spm1Δ* mutants had a similar behavior in some stress-inducing media suggested that Cfh3p could be part of the Spm1/Pmk1 MAP kinase pathway (Toda *et al.*, 1996, Zaitsevskaya-Carter & Cooper, 1997, Madrid *et al.*, 2006). On the other hand, the fact that the *cfh3Δ* cells did not show an aberrant cytokinesis when it was suffering stress seemed to argue against this hypothesis. In order to analyze the relationship between Cfh3p and this MAP kinase pathway in more detail we performed the experiments described below.

### 11.1. Co-immunoprecipitation between Cfh3p and Rho2p or Cdc42p

Since Rho2p acts upstream of Pmk1 MAPK signaling pathway (Ma *et al.*, 2006) and Cdc42p is also known to be a regulator for Spm1p MAPK (Merla & Johnson, 2001), we wanted to know whether there was any physical contact between those protein (Rho2p or Cdc42) and Cfh3p. To check this possibility, we performed co-immunoprecipitation assays between those proteins and Cfh3p using strains carrying a GFP-tagged *cfh3*$^+$ gene and an HA-tagged *rho1*$^+$ or *cdc42*$^+$ genes, or both. As seen in figure 40 A, HA-Rho2p was detected in anti-GFP immunoprecipitates from the strain bearing both HA-Rho1p and GFP-Cfh3p, but not from the control strains, pointing to a physical interaction between Cfh3p and Rho2p. On the contrary, Cdc42p was not detected in the immunoprecipitates (results not shown).

### 11.2. Analysis of the vic phenotype

One experimental procedure directed to know

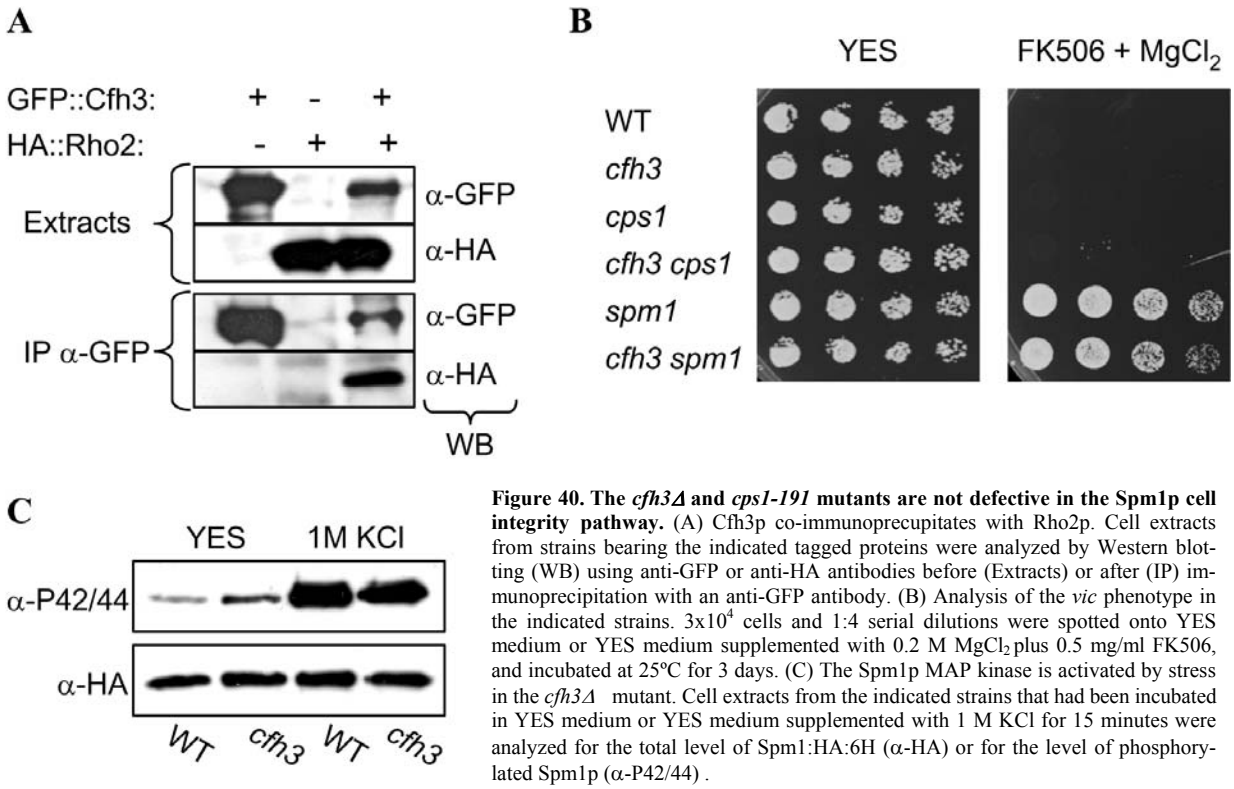



**Figure 40. The *cfh3Δ* and *cps1-191* mutants are not defective in the Spm1p cell integrity pathway.** (A) Cfh3p co-immunoprecupitates with Rho2p. Cell extracts from strains bearing the indicated tagged proteins were analyzed by Western blotting (WB) using anti-GFP or anti-HA antibodies before (Extracts) or after (IP) immunoprecipitation with an anti-GFP antibody. (B) Analysis of the *vic* phenotype in the indicated strains. $3x10^4$ cells and 1:4 serial dilutions were spotted onto YES medium or YES medium supplemented with 0.2 M $MgCl_2$ plus 0.5 mg/ml FK506, and incubated at 25ºC for 3 days. (C) The Spm1p MAP kinase is activated by stress in the *cfh3Δ* mutant. Cell extracts from the indicated strains that had been incubated in YES medium or YES medium supplemented with 1 M KCl for 15 minutes were analyzed for the total level of Spm1:HA:6H (α-HA) or for the level of phosphorylated Spm1p (α-P42/44) .

whether a gene works in the *spm1*$^+$ pathway is to check if the mutants in that gene exhibit the so-called *vic* phenotype: viability in the presence of immunosuppressant (0.5 µg/ml FK506) and chloride (0.2 M $MgCl_2$), which is characteristic of mutants in the components of that pathway (Ma *et al.*, 2006). The *cfh3Δ* mutants did not show the *vic* phenotype, indicating that *cfh3Δ* is not a component of the Spm1/Pmk1 MAPK signaling pathway (figure 40 B).

### 11.3. Activation of Spm1/Pmk1p

The level of activation of the Spm1p kinase was assessed in a WT and a *cfh3Δ* strains carrying a *pmk1-HA6H:ura4*$^+$ modified gene as explained in Materials and methods. Both strains were incubated under standard laboratory conditions (YES medium) or under stress conditions (1 M KCl) for 15 minutes at 28ºC. The level of activation of the kinase was estimated by detecting the amount of phosphorylated protein respect to the input of Spm1 protein. As shown in the figure 40 C, in the absence of *cfh3*$^+$, Spm1p was efficiently phosphorylated when the cells suffered an osmotic shock in both, the WT and the *cfh3Δ* strains. In addition, we detected that the basal level of activation of the Spm1p kinase was slightly higher in the *cfh3Δ* mutant than in the WT strain. This could be explained because the defect in the β-glucan synthesis in this mutant was probably producing some stress to the cells.

## 12. Chs4p does not complement the phenotype of *cfh3Δ* cells

*CHS4* is a gene from *S. cerevisiae* that shows 24% identity to *cfh3*$^+$, so we wondered whether this gene was able to complement the phenotype of the *cfh3Δ* mutant. To address this question, the WT and the *cfh3Δ* strains were transformed with the pREP3X vector or the pREP3X+*CHS4* plasmid. In order to determine cell viability under stress conditions, two sets of MM without leucine plates were prepared and one of them was supplemented with 0.2 M $MgCl_2$. In addition, plates from each set were supplemented with thiamine (repressing conditions for the expression of *CHS4*) or not (derepressing conditions). In each case, 3 x $10^4$ cells and three 1:4 serial dilutions were spotted onto the corresponding plates that were incubated at 32ºC. As shown in figure 41, all strains were able to grow in the absence of $MgCl_2$ independently of the presence of thiamine. However, the *cfh3Δ* cells carrying the empty vector or the *CHS4* overexpression plasmid showed hypersensitivity to 0.2 M $MgCl_2$ even under derepressing conditions, indicating that *CHS4* was not able to complement the phenotype of *cfh3Δ* mutants.

With the purpose of knowing whether Chs4p was not a functional homologue of *cfh3*$^+$, or if this protein was not able to complement the phenotype of *cfh3Δ* mutants because it was not properly expressed

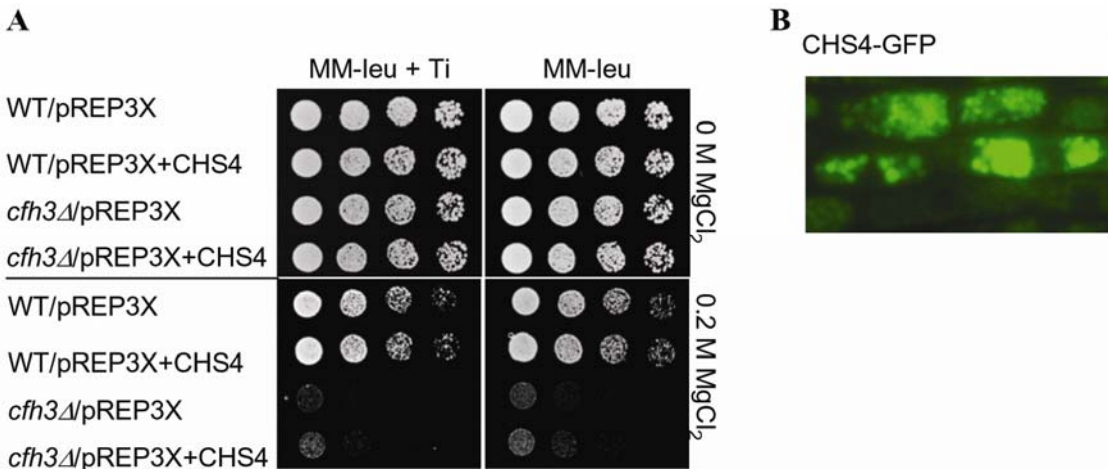



**Figure 41. Chs4p does not complement the *cfh3Δ* muation.** (A) The WT or *cfh3Δ* strains were transformed with the indicated plasmids. 3x$10^4$cells and 1:4 serial dilutions were spotted onto selective medium supplemetd or not with $MgCl_2$ in the presence of thiamine (repression) or in its absence (derepression). (B) Localization of GFP-Chs4p in *S. pombe*.

or localized in the fission yeast, the *chf3Δ* mutant was transformed with a pREP3X+*CHS4-GFP* plasmid, in which Chs4p was fused to the GFP. As shown in the figure 41, Chs4p was not able to localize properly and the fluorescent signal accumulated in some structures that probably corresponded to the vacuoles, indicating that Chs4p was not stable in *S. pombe*.

## 13. Analysis of the functionality of Cfh3 proteins lacking the SEL1 domains or the prenylation site

Cfh3p is a member of a family of 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 (Karpenahalli *et al.*, 2007). Additionally, like many member from this family, Cfh3p has a C-terminal prenylation motif (CIIS. See figure 42 A for a scheme of the Cfh3p structure). We analyzed the stability and localization of the full-length Cfh3 protein, Cfh3 proteins in which all or several SEL1 domains had been deleted, and a Cfh3 protein in which the prenylation sequence had been eliminated, all fused to the HA or the GFP, respectively. We found that elimination of the SEL1 domains had no apparent effect in the stability or the localization of Cfh3p (figures 42 B and C, and results not shown). We checked the relevance of these domains for Cfh3p function by analyzing the growth capacity of cells carrying different mutated forms of Cfh3p on minimal medium plates supplemented with 0.2 M $MgCl_2$ incubated at 32ºC. As shown in figure 42 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 $MgCl_2$.

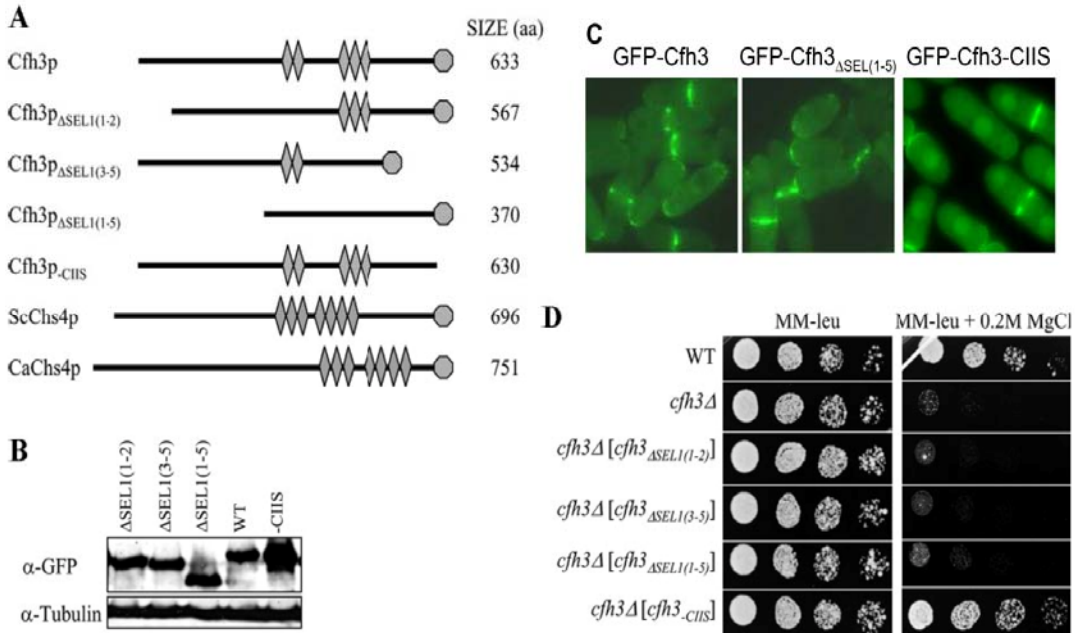



**Figure 42**. **The SEL1 domains are required for Cfh3p function.** (A) Schematic representation of the full-length Cfh3 protein, or different truncated versions of this protein, where the grey diamonds represent the SEL1 domains and the grey circle represents the prenylation motif. The *Saccharomyces 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

# Discussion

In this work we have characterized the function of the Cfh3 protein. It has been suggested that Cfh3p/Chr4p was a regulator of the chitin synthase-like protein Chs2p (Matsuo *et al.*, 2004). Although we cannot exclude this possibility completely, the following results disagree with this conclusion: i) we have observed that Chs2p localized at internal vesicles in both, the WT and the *cfh3Δ* strain, depending on the culture conditions (figure 43); ii) we have not found any genetic interaction between the *cfh3Δ* and the *chs2Δ* mutants; iii) localization of Chs2p depends on the myosin component of the CAR (Martin-Garcia & Valdivieso, 2006) but that of Chs3p does not; iv) the *chs2Δ* mutant shows a genetic interaction with the myosin mutants (Martin-Garcia & Valdivieso, 2006) but the *cfh3Δ* mutant does not; v) localization of Chs2p under stress conditions is the same in the WT and the *cfh3Δ* mutant; vi) the *chs2Δ* cells are not sensitive to stress conditions.

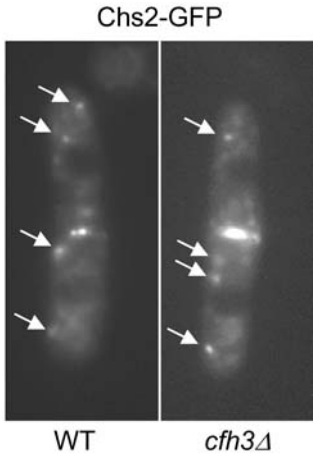



**Figure 43. Localization of Chs2-GFP**. Cells from the WT and *cfh3Δ* strains bearing Chs2-GFP were photographed. The arrows point to cytoplasmic fluorescent dots.

Our results point to a role of Cfh3p in regulating the glucan synthase Bgs1p. Deletion of the *cfh3*$^+$ gene alone or simultaneous deletion of the four *cfh*$^+$ genes produced no obvious phenotype. However, *cfh3*$^+$ is slightly cell-cycle regulated with a peak of expression at the end of mitosis (see http://www.sanger.ac.uk/PostGenomics/S_pombe/projects/cellcycle/), *cfh3*$^+$ overexpression produced an aberrant cytokinesis, and Cfh3p localizes at the septal area of the cells (Matsuo *et al.*, 2004 and this work). These data suggested that Cfh3p might play a role in cytokinesis. In order to pinpoint the time at which Cfh3p might be exerting its function, we followed several experimental approaches: i) analyzing the localization of proteins involved in cytokinesis in cells overexpressing *cfh3*$^+$; ii) analyzing the phenotype of *cfh3*$^+$ overexpression in mutants lacking some proteins involved in cytokinesis; iii) analyzing the localization of Cfh3p in mutants altered in different steps of cytokinesis; iv) analyzing the localization of proteins involved in cytokinesis in the *cfh3Δ* mutant; v) analyzing the genic interactions between the *cfh3Δ* mutant and mutants affected in different steps of cytokinesis.

We found that the distribution of all the proteins analyzed, which participated in different steps of cytokinesis, was abnormal in cells overexpressing *cfh3*$^+$, suggesting that Cfh3p might contact with many proteins and might be a component of a multiprotein complex. The phenotype of *cfh3*$^+$ overexpression required an active SIN pathway (necessary for ring contraction and septum synthesis; see Krapp *et al.*, 2004, Krapp & Simanis, 2008 for reviews), the protein Cdc15p (a component of the CAR; Fankhauser *et al.*, 1995) and Bgs1p (the glucan synthase required for the synthesis of the primary septum; Cortes *et al.*, 2007). Cfh3p required the SIN pathway and Cdc15p to localize. All the cytokinesis proteins analyzed were able to localize at the septal area in a *cfh3Δ* mutant, although some of them exhibited an abnormal appearance. Finally, we found that a *cfh3Δ* mutant had a genic interaction with *cps1-191* (carrying a mutation in the glucan synthase *bgs1*$^+$), *cps8-188* (carrying a point mutation in the *act1*$^+$ gene, coding for actin; Ishiguro & Kobayashi, 1996), *cdc15-140* (carrying a point mutation in the *cdc15*$^+$ gene), and with *cdc14-118* (bearing a mutation in a gene from the SIN pathway). We found a genetic interaction with the SIN mutant *cdc14-118*, but not with the SIN mutant *cdc11-119*. The same result had been obtained when genetic interactions for the *chs2Δ* mutant were analyzed (R. Martin-Garcia and M.H. Valdivieso, unpublished results). This different behavior in the two SIN mutants could be explained if the scaffold protein Cdc11p was not as required as the Sid1 kinase-associated protein Cdc14p to regulate the Morphogenesis Orb6 network (MOR), which is involved in polar growth and cell separation (Kanai *et al.*, 2005, Mendoza *et al.*, 2005). Altogether, the results described above pointed to a role of Cfh3p in ring assembly or contraction, or in septum synthesis.

We have found that in a *cfh3Δ* mutant there is a

small reduction in the synthesis of β-glucan. This reduction is similar to that found in the glucan synthase mutant *cps1-191*. Although we cannot rule out completely some regulation of other glucan synthases, the facts that a mutant in the *bgs4*$^{+}$ glucan synthase is not sensitive to stress, that a low concentration of Calcofluor cannot stain the septa in the *cfh3Δ* mutant, and that the morphology of the *cfh3Δ cps1-191* cells is similar to that of the *bgs1Δ* cells maintained alive with an osmotical support (Cortes *et al.*, 2007) suggested that the defect in the β-glucan synthesis in the *cfh3Δ* cells was due to a defect in the activity of Bgs1p. A defect in this glucan synthase is expected to result in a small decrease in the β-glucan content, since Bgs1p is responsible for the synthesis of linear β-glucan (Cortes *et al.*, 2007), which is a minor component of the cell wall (Humbel *et al.*, 2001).

*cfh3Δ*, *cps1-191* and *cfh3Δ cps1-191* strains have a reduced viability when the media are supplemented with some stress-inducing compounds. In these mutants, a significant number of cells exhibit abnormal contractile rings and this number increase when the cells are under stress. However, it seems that the cells do not die because of an abnormal cytokinesis, as it has been described for the *spm1Δ* mutants (Zaitsevskaya-Carter & Cooper, 1997), but because of a reduced cell wall synthesis. A reduction in the total cell wall synthesis has also been observed in different strains grown in the presence of sorbitol (Cortes *et al.*, 2005, Cortes, 2006). A feeble Bgs1 activity would produce a further defect with respect to that produced by the stress in the WT strain. Thus, in the double *cfh3 Δ cps1-191* mutant cells grown under stress conditions the amount of β-glucan is 50% of the value of the WT cells grown under standard conditions. This strong defect would be deleterious for the cells.

Cfh3p was associated with the Cdc15p ring protein and with the glucan synthase complex under standard or stress conditions. Under normal laboratory conditions, most of Bgs1p seemed to be localized at the plasma membrane in the absence of *cfh3*$^{+}$. Accordingly, the cells grew normally and only showed a small reduction in the synthesis of linear β-glucan and a small increase in the number of defective rings respect to the WT strain. It has been described that when the cells are exposed to some stress condition, there is a change in the physical state and molecular interactions in the membrane bilayer, influencing lipid packing and dynamics (Kinnunen, 2000). Transmembrane proteins are then expected to assume new conformations and associations, and to change their interactions with membrane lipids (Poolman *et al.*, 2004). Under these circumstances, the Bgs1p glucan synthase was not observed at the plasma membrane if Cfh3p was absent. The effect of the stress was abolished when endocytosis was prevented. Thus, it seems that Cfh3p could act as a scaffold required to stabilize Bgs1p at the plasma membrane so the synthesis of β-glucan is ensured even if the environment is unfavorable. The cells have a higher requirement for the synthesis of linear β(1,3)glucan at the septum. Therefore, the strong interaction of Cfh3p with the CAR would guarantee that the primary septum can be synthesised correctly.

In agreement with a relationship between the amount of Cfh3p and the presence of Bgs1p at the membrane, we have found that when *cfh3*$^{+}$ is overexpressed there is a higher amount of Bgs1p that can be observed around the cell. However, we cannot rule out the possibility that Cfh3p is also producing some post-translational modification in Bgs1p required for its activity. Moreover, it is possible that Cfh3p does not regulate Bgs1p endocytosis but its conformation so in its absence, and under stress conditions, the conformation of Bgs1p would change and this phenomenon would somehow avoid the emission/observation of the GFP signal. We tried to discern between these possibilities by performing subcellular fractioning with discontinuous sucrose gradients. The technique did not produce the expected results, since in the WT strain grown under standard conditions it was difficult to detect Bgs1 protein in the heaviest (membrane-associated) fractions.

We have found that in the *cps1-191* mutant contractile rings are also defective, pointing to an implication of Bgs1p in the control of CAR morphology. In the presence of an osmotic or nutritional stress, the number of cells with an abnormal contractile ring increased dramatically in the *cfh3Δ*, the *cps1-191* and *cfh3Δ cps1-191* cells. Since these conditions seemed to reduce the activity of Bgs1p (and maybe other glucan synthases), the high frequency of cells with abnormal

rings under stress conditions is possible the consequence of an enhanced defect in the glucan synthase in those strains.

It will be interesting to determine whether the defective rings, which can be observed even in the WT strain under stress conditions, are produced as a consequence of the synthesis of abnormal primary septa or if the Bgs1 protein itself plays a direct role in the stability of the rings. The fact that the number of defective rings was high after a short osmotic shock (15 minutes) points to the latter hypothesis. It is known that an osmotic shock results in cell shrinkage. Probably, the change in the cell volume after the osmotic shock produces a deformation in the membrane that would explain the deformed septal area observed in the WT and mutant strains under these conditions. It is possible that this deformation affects the conformation of Bgs1p, a protein with 13 transmembrane domains, and that this can lead to a reduced activity of the protein and/or to a modified interaction with the CARs, which would result in the observed defects. It has also been described in budding and fission yeast that the osmotic shock leads to a depolarization of the actin patches, and that after a time in the hyperosmotic environment, the cells adapt to the new conditions and relocalize the actin patches (Brewster & Gustin, 1994, Bao *et al.*, 2001). The reason for the different behavior of Bgs1p in the WT or the *cfh3Δ* strains treated with osmotic stress-inducing compounds does not seem to be a consequence of a different behavior of the actin cytoskeleton in these cells, because under our conditions the actin patches were still polarized in both strains. We found that the initial shock had an effect in the CAR morphology but that after a longer treatment (2 hours) the rings were normal in all the strains. In this way, the contractile rings, which are specifically assembled for cytokinesis, seem to behave in a similar way to the actin cytoskeleton in response to an osmotic shock.

Although it seems that the defect in ring stability/structure observed in the *cfh3Δ* mutant is a consequence of the defect in the Bgs1 glucan synthase, we cannot exclude a specific role of Cfh3p in the morphology of the rings because the *cfh3Δ* mutant shows a stronger defect than the WT strain, and the double *cfh3 Δ cps1-191* mutant shows a stronger defect than that of the single *cps1-191* mutant. In agreement with this hypothesis, overexpression of the *cfh3*$^+$ gene leads to a strong defect in cytokinesis that is not observed in cells overexpressing *bgs1*$^+$ (figure 44 and Cortes, 2006).

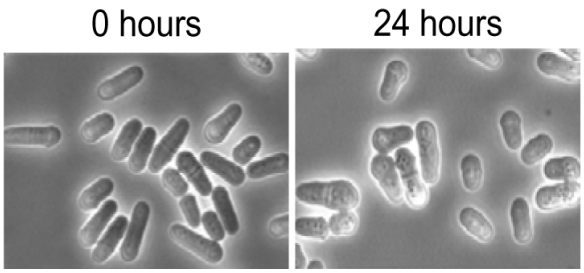



**Figure 44. Phenotype of *bgs1*$_+$ overexpression**. Cells expressing the *bgs1*$^+$gene under the control of the thiamine-repressible promoter *nmt1*$^+$ were incubated in the absence of thiamine for the indicated times and photographed (This picture is a generous gift of J. C. Ribas).

However, there is another way to explain the different phenotype in the strains under study: in the WT strain, Bgs1p arrives to the membrane and remains there the time required to exert its normal activity, and to synthesize β-glucan at a normal rate. In the *cfh3Δ* mutant, a robust Bgs1p is delivered to the membrane, so it is able to act properly for a time, but this protein is endocyted faster than in the WT strain; this produces a feeble activity resulting in some cell defects. In the *cps1-191* mutant, the protein delivered to the membrane is defective so, although it stays at the membrane for a normal time, it produces a weaker activity than that of the WT strain. Finally, in the *cfh3 Δ cps1-191* double mutant, a defective Bgs1 protein would be delivered to the membrane and endocyted faster than in the single *cps1-191* mutant; as a result, the Bgs1-dependent activity would be weaker than in the single mutants, accounting for the strong defects detected in this strain.

The fact that in the *cfh3Δ* strain Bgs1p would be able to exert its function for a time would explain the mild phenotype observed in this mutant. This phenotype was not aggravated in mutants lacking other *cfh*$^+$ genes or even the four of them, indicating that they do not collaborate with *cfh3*$^+$ in its function. In any case, minor growth impairments under stress conditions have been described before for other regulators

of proteins required for viability. Thus, deletion of the Skb5 or the Skb1 proteins, which are *bona fide* regulators of Shk1/Pak1/Orb2p (a essential PAK-related kinase involved in Ras/Cdc42 signaling), does not lead to any obvious phenotype when the cells are incubated under normal laboratory conditions, and they only show a slow growth when the cells are incubated in minimal medium supplemented with 1.5 M KCl at 30ºC (Yang *et al.*, 1999, Bao *et al.*, 2001).

The *cfh3Δ* cells showed impaired growth under stress conditions, which suggested that it could be part of the Spm1/Pmk1 MAP kinase pathway (Toda *et al.*, 1996, Zaitsevskaya-Carter & Cooper, 1997, Madrid *et al.*, 2006). There are some data suggesting this possibility: i) *cfh3Δ* and *spm1Δ* mutants have a similar behavior in some stress-inducing media; ii) Cfh3p co-immunoprecipitates with Rho2p, a GTPase that regulates the Spm1/Pmk1 kinase (Ma *et al.*, 2006). However, the following results argue against this hypothesis: i) Cfh3p does not co-immunoprecipitate with Cdc42p, another GTPase that regulates the Spm1/Pmk1 kinase (Merla & Johnson, 2001); ii) the *cfh3Δ* mutants do not show an altered cytokinesis when they grow under stress conditions; iii) the *cfh3Δ* mutants do not show the *vic* phenotype, characteristic of mutants in the Spm1/Pmk1 MAPK signaling pathway (Ma *et al.*, 2006); iv) in the absence of $cfh3^{+}$, Spm1p is efficiently phosphorylated when the cells suffer an osmotic shock.

We detected that the basal level of activation of the Spm1p kinase was slightly higher in the *cfh3Δ* mutant than in the WT strain. This can be explained because the defect in the β-glucan synthesis in this mutant is probably producing some stress to the cells. Also, the co-immunoprecipitation between Rho2p and Cfh3p can be explained because Rho2p regulates α-glucan synthesis (Calonge *et al.*, 2000) and in the absence of Cfh3p there is an increase in the synthesis of this polymer, which suggests that Cfh3p could associate with the α-glucan complex and regulate it.

Cfh3p shows a significant degree of similarity with Chs4p, a protein that regulates the chitin synthesis mediated by the chitin synthase Chs3p in *S. cerevisiae* (DeMarini, 1997, Trilla *et al.*, 1997, Ono N, 2000, Grabinska *et al.*, 2007, Reyes *et al.*, 2007). Chs3p regulation 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 the septin ring through Bni4p (DeMarini, 1997, Reyes *et al.*, 2007), but it also acts as a biochemical activator, since *CHS4* overexpression leads to an increase in the chitin synthase activity and chitin synthesis (Trilla *et al.*, 1997, Sudoh, 1999, Ono N, 2000, Reyes *et al.*, 2007). Respect to its specific mechanism of action, it seems that a protein-protein interaction is required (DeMarini, 1997, Ono N, 2000) and that it can be exerted at different levels. It has been shown that in a *chs4Δ* mutant, Chs3p is endocyted faster than in the WT strain (Reyes *et al.*, 2007). However, a stabilization of the chitin synthase in the plasma membrane in the absence of Chs4p does not produce an increase in the amount of active Chs3p, so it seems that Chs4p mediate some posttranslational modification in Chs3p (Reyes *et al.*, 2007). In *S. pombe*, there is no chitin synthesis during vegetative growth (Kreger, 1954, Horisberger & Rouvet-Vauthey, 1985, Sietsma & Wessels, 1990, Arellano *et al.*, 2000) β-glucan being the main cell wall component, there is no Bni4p homologue, and septins are involved in cell separation but not in septum synthesis (An *et al.*, 2004, Martin-Cuadrado *et al.*, 2005). In this organism, there are four proteins with significant similarity to Chs4p (Matsuo *et al.*, 2004 and our unpublished results). Our results show that one of these proteins (Cfh3p) plays a role in the regulation of glucan synthesis, in agreement with the idea that in *S. pombe* the Chs-like proteins have maintained a general function not related to chitin synthesis, as it has been reported for Chs2p and Cfr1p (Martin-Garcia *et al.*, 2003, Cartagena-Lirola *et al.*, 2006, Martin-Garcia & Valdivieso, 2006). In this case, the function would be to regulate the stability at the plasma membrane of an enzyme required for the cell wall synthesis at the division septum and/or to modify this protein. Anyway, the specific requirements for this regulation must be different in different organisms, since Chs4p cannot localize properly in the fission yeast.

The most relevant features in the Cfh3p molecule are the presence of a potential prenylation site and the presence of five SEL1 repeats. We found that the prenylation motif is not essential for Cfh3p function. Similar results were obtained for the *S. cerevisiae*

Chs4 protein (DeMarini, 1997, Grabinska *et al.*, 2007, Reyes *et al.*, 2007). On the contrary, 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 (DeMarini, 1997, Ono N, 2000, Reyes *et al.*, 2007). The region of the *Candida albicans* Chs4 protein that contains the SEL1 domains is able to complement the *S. cerevisiae chs4Δ* mutant (Sudoh, 1999). 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 in multiprotein complexes involved in different cellular processes such as cell cycle control or ER-associated protein degradation (Mittl & Schneider-Brachert, 2006). 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.

# Conclusions

1. Cfh3p is a posttranslational regulator of the β(1,3)glucan synthase Bgs1p.

2. Cfh3p and Bgs1p are required to ensure the stability of the contractile acto-myosin ring.

3. The function of Cfh3p is more necessary when the cells are under stress conditions.

4. In *Schizosaccharomyces pombe* glucan synthesis is reduced when the cells are undergoing stress .

# Materials & Methods

## 1. Strains

The list of the strains used in this work is shown in table 3 in the appendix.

## 2. Culture media and growth conditions

All culture media were prepared with distilled water (Milli-Rho) and sterilized by autoclaving. The corresponding solid media were prepared by adding 2% or 3% agar, depending on the cases.

### 2.1. Growth of *Escherichia coli*

*E.coli* strains were grown at 28ºC and/or 37ºC in media and conditions described by (Sambrook *et al.*, 1989, Sambrook, 2001), either on plates or in liquid media. In order to select the resistant clones to antibiotics, the corresponding medium was supplemented with ampicillin (100 µg/ml) or kanamycin (25 µg/ml). See the composition of media in the appendix.

### 2.2. Growth of *Schizosaccharomyces pombe*

The culture media used for the growth and maintenance of *S. pombe* have been described before (Moreno *et al.*, 1991). Additionally, all general techniques for working with fission yeast can be found at: http://www.burnham.org/labs/wolf/Protocols/Protocols/Fission%20Yeast/Nurse%20Lab%20Manual.htm . See also the Apendix.

#### 2.2.1. Vegetative growth

Unless stated otherwise, cells were analyzed when they were in the exponential phase of growth. For vegetative growth of *S .pombe*, rich media (YES) or minimal media (EMM), whose composition is detailed in the appendix, were used. When it was necessary, minimal media were supplemented with 225 mg/l of adenine, histidine, leucine, and/or uracil, according to the requirements of the strains. These supplements were added from sterilized concentrated solutions. *S. pombe* strains were incubated at different temperatures, depending on the strains and experiments, although the optimal temperature of growth for the wild type (WT) strain was 32ºC. Some ts strains were grown at 25ºC or 37ºC, depending on the nature of the experiments. For liquid cultures, cells were grown in flasks shaken at 250 rpm, and the cellular growth was controlled by determining the optical density (O.D.) of the culture at 600 nm in a spectrophotometer (Hitachi U-2001). It has been previously determined that for *S. pombe,* an $O.D._{600} = 1$ is equivalent to $2 \times 10^7$ cells/ml. For overexpression experiments using the $nmt1^+$ promoter in the pREP plasmids (Maundrell, 1993), cells were grown in EMM medium containing the appropriate supplements including 15 µM thiamine, which represses the expression of this promoter. In order to derepress the expression from the promoter cells were harvested, washed 3 times with water and resuspended in the same media without thiamine at a determined O.D. Usually, the initial $O.D._{600}$ was 0.01, so that after the induction time this O.D. did not go above a value of 1. For phenotype or protein analysis cells were grown in the medium devoid of thiamine for 20-22 hours. In order to select clones resistant to Geneticine, YES solid medium was supplemented with Geneticine Sulphate (G-418; Life Technologies) at a final concentration of 120 µg/ml.

#### 2.2.2. Mating and sporulation

In order to construct double mutants or to combine a certain mutation with a chromosome-integrated tagged protein, genetic crosses involving the parental strains of interest were carried out. To do this, the same amount of cells from each parental strain (belonging to the complementary sexual types, $h^+$ and $h^-$) were mixed on a SPA (appendix) plate and incubated at 28ºC (or 25ºC in the case of thermo-sensitive strains) for 2-3 days. When asci were mature, random spore or tetrad analysis were performed to separate the spores. For the selection of spores at random, when the asci were mature either independent spores were micromanipulated ("organized random spore" analysis) or a small sample of cells was resuspended in 1 ml of water containing 5 µl of the "*Helix pomatia* juice" extract enzyme (Biosepra), which digests the *S. pombe* cell wall from the cells, but not from the spores. The mixture was incubated over night in a tube rotator at 28ºC, in order to facilitate the liberation of spores. On the following day, the number of spores per ml was determined by counting in a Thoma chamber and dilu-

tions were preformed so that 300-500 spores were plated on YES plates. To select the clones of interest, replicas were performed on different media and incubated at different temperatures, if ts strains were involved.

### 2.2.3. Special growth conditions

Media supplemented with salts

To analyze the growth of *S. pombe* strains of under stress conditions, YES medium was supplemented with different concentrations of salts, and the plates were incubated at different temperatures.

KCl was used at 0.6 M, 1.0 M, 1.2 M, 1.4 M, and 1.5 M.

NaCl was used at 0.1 M, and 0.15 M.

$MgCl_2$ was used at 0.05 M, 0.075 M, 0.1 M, 0.125 M, 0.15 M, 0.175 M, and 0.2 M.

Media supplemented with 0.25µg/ml FK506 and different concentration of $MgCl_2$

In order to analyze the presence of a *vic* phenotype (viable in the presence of immunosuppressant and chloride), FK506 (LC Laboratories), an inhibitor of calcineurin, was added at a concentration of 0.5 µg/ml to YES medium supplemented with the following concentrations of $MgCl_2$: 0.075 M, 0.1 M, 0.125 M, 0.15 M, and 0.2 M. The growth was compared with the growth on YES medium supplemented with the same concentration of $MgCl_2$ without FK506.

Media supplemented with hydrogen peroxide ($H_2O_2$)

YES medium supplemented with 0.1 mM, 0.2 mM, 0.5 mM and 1.0 mM $H_2O_2$ was used in order to analyze the growth in the presence of an oxidative stress.

Media supplemented with sorbitol

1.2 M sorbitol was used as an osmotic stabilizer and also to produce an osmotic shock to the cells.

Media supplemented with Caffeine

To analyze the growth of certain strains in the presence of caffeine, YES medium was supplemented with this compound at 5 mM, 6 mM, and 7 mM.

Media supplemented with LatrunculinA

Latrunculina A was used with the purpose of analyzing the localization of Cfh3p after disrupting the actin cytoskeleton, or with the purpose of studying if certain mutants have some problem in the actin cytoskeleton. This compound is a toxin, obtained from the sponge *Latrunculia magnifica* and inhibits the polymerization of actin due to its union to actin G monomers. To test the dependency of protein localization with respect to actin, the drug was added at a 100 µM final concentration to a liquid culture for 15 minutes. The stock solution was at 5 mM in DMSO. These tests were performed in duplicate, using a culture treated with DMSO at 0.5% (v/v) as a control. To test growth in the presence of low doses of Latrunculina A, the drug was used at 0.1 µM, 0.2 µM, 0.3 µM and 0.4 µM on YES solid medium.

Media supplemented with antifungal drugs that affect the cell wall

Calcofluor, Caspofungin or Enfumafungin, were added to YES plates to analyze the effect of antifungal drugs on the cell wall of certain strains.

Calcofluor was used at 1.0 mg/ml and 2.0 mg/ml.

Caspofungin was used at 0.75 µg/ml, 1.5 µg/ml, and 2.0 µg/ml.

Enfumafungin was used at 3.0 µg/ml, 5.0 µg/ml, and 7.0 µg/ml.

## 3. Molecular and genetic manipulations

### 3.1. Plasmid used

*E. coli* and *S. pombe* vectors used in this work are shown in table 4 in the appendix.

### 3.1.1. *E.coli* Vectors

pBluescript II KS+ and SK+ (Stratagene)

These plasmids are autoreplicative in *E. coli* because they have an origin of replication for this bacterium. They have a size of 2958 base pairs (bp). They carry a gene that confers resistance to Ampicillin and a fragment of the *E. coli* Lac operon, which codes for the α-peptide of the β-galactosidase enzyme so when inserts are cloned into the Multi Cloning Site (MCS) this fragment is broken and there is no β-galactosidase activity (which allows the selection of the plasmids with insert because the colonies are white, while the colonies with the empty vector are blue in the presence of the X-gal substrate). The MCS, also called polylinker, is a 108 bp DNA fragment that carries the sites for 21 restriction enzymes that are unique in the vector. This fragment is in frame with the α-peptide of the β-galactosidase enzyme. Both vectors differ in the orientation of the MCS. These vectors also have sequences that can be recognized by the M13K07 phage which allows the recovery of single strand DNA (ssDNA) using this phage.

pGEM-T (Promega)

This is a plasmid with a 3003 bp size, and is commercially linearized with an additional thymin in the ends that facilitates the insertion of fragments generated by PCR. Like the pBluescript II vectors, pGEM-T carries a gene that confers resistance to Ampicillin and a fragment of the *E. coli* Lac operon that allows selecting the recombinant clones by alpha complementation (white/blue). pGEM-T has 13 unique sites in the MCS. Similar to the pBluescript II vectors, pGEM-T has also a sequence that can be recognized by M13K07 phage which allows getting ssDNA using this phage.

### 3.1.2. *S. pombe* Vectors

The plasmids used for *S. pombe* share a series of common characteristics that basically consist of: an origin of replication, a bacterial selection marker (generally a gene for the resistance to an antibiotic), a yeast selection marker (normally a metabolic marker), and an **a**utonomous **r**eplicative **s**equence (ARS). Unlike *S. cerevisiae* plasmids, centromeric plasmids (monocopy) do not exist for the fission yeast because the centromere in the fission yeast is too big to be included in the plasmids. An alternative is to use integrative plasmids, lacking an ARS. Linearization of the plasmid allows the stable integration in the genome of fission yeast by homologous recombination.

pAL-KS$^+$

It is a mulicopy plasmid with 6,300 bp size. It has the *ars1*$^+$ sequence from *S. pombe* that allows replication so it can be maintained in the cells independently of the chromosome. Every cell has several copies of the plasmid. It is a derivative of pBluescript II KS$^+$, so it has the same properties. The selective marker is the *LEU2* gene from *S. cerevisiae*, which is able to complement the *S. pombe leu1-32*mutation. The MCS includes the sites for 11 restriction enzymes that are unique in the vector.

pAU-KS$^+$

It is 5.6 kb in size and has the same characteristics as pAL but the selection marker is the *URA3* gene from *S. cerevisiae* that is able to complement the *ura4-Δ18* mutation from *S. pombe.*

pREP plasmids

These vectors were obtained from the *E. coli* plasmid pUC119. They confer resistance to Ampicillin and are autonomous in the bacteria. The selective marker for *S. pombe* is the *LEU2* gene from *S. cerevisiae*. They carry the *nmt1*$^+$ promoter, which is repressible by thiamine. There are three versions of the promoter, according to the strength of the expression that they produce. PREP3X is the strongest, 41X is medium strength and pREP81X is the weakest. They have a MCS downstream from the *nmt1*$^+$ promoter. Notice that the polylinker in the 40-X and 80-X series is not the same as the polylinker in the 3X and 4X series (Forsburg, 1993, Maundrell, 1993). Some derivatives of these plasmids that carry different selection markers and other MCS (Moreno *et al.*, 2000) were used in this work.

pJK148

It is an integrative plasmid with 5343 bp size and a MCS. It does not have an ARS sequence. The only way to be maintained for the cell is to be integrated at the chromosome. We can increase the efficiency of integration by digesting with a unique enzyme that cuts in a yeast sequence (in the gene of in-

terest or the selection marker). The selection marker is the *leu1*$^+$ gene from *S. pombe* (McLeod *et al.*, 1987, Keeney & Boeke, 1994).

### 3.2. General techniques

Bacteria and yeast transformations, DNA extraction, PCR, site-directed mutagenesis, and other general DNA manipulation were performed by standard procedures, according to (Rothstein, 1983, Kunkel *et al.*, 1987, Sambrook *et al.*, 1989, Moreno *et al.*, 1991, Sambrook, 2001).

### 3.3. Plasmid construction

#### 3.3.1. Cloning of the *cfh3*$^+$ gene

The *cfh3*$^+$ gene was cloned from the genome of an *S. pombe* WT strain by the "Gap Repair" technique (Orr-Weaver *et al.*, 1983). This technique allows the transference of a DNA fragment from the genome to a replicative plasmid. The plasmid must carry the DNA fragments flanking the sequence of interest in order to allow the homologous recombination between these regions in the plasmid and the genome. The plasmid is digested with restriction enzymes to linearize it so there is a "gap" in-between the *S. pombe* DNA fragments that will be filled in by the genomic sequence through an homologous recombination process. This event allows the plasmid to replicate in the cells so colonies can grow on selective plates. Finally, the plasmid is recovered from the yeast cells and amplified by introducing it in *E. coli* by transformation. In our case, the *cfh3*$^+$ gene was cloned as follows:

**1.** Oligos cfh3Kpn (5'-atg tcc agc GGT ACC cag cct ttt tgg gat gaa aga-3') and cfh3XhoI (5'-tcc tca gtg CTC GAG ata tcg tct ttt aag tca att-3') were used to amplify a 1 kb-DNA fragment, flanked by the *Kpn*I and *Xho*I restriction sites, from the genome, by PCR. This fragment, which is located about 1 kb upstream of the initial ATG of the ORF in the genome (see figure 45) was cloned into the *Kpn*I and *Xho*I restriction sites of the MCS in the pAU plasmid. The resulting plasmid was named pAU+5'cfh3.

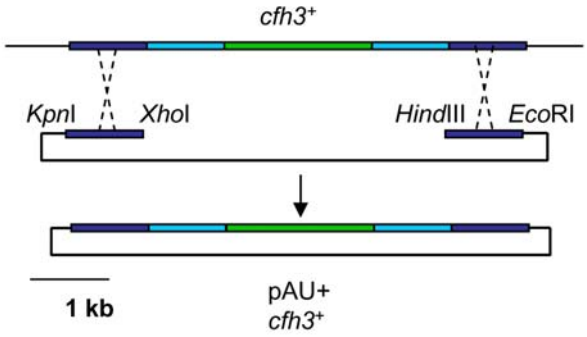



**Figure 45. Cloning of *cfh3*$^+$ by *Gap Repair*.** The *cfh3*$^+$ gene was cloned from the genome by homologous recombination between the genomic DNA and the *Kpn*I-*Xho*I and *Hin*dIII-*Eco*RI DNA fragments cloned in the pAU vector. These fragments had been amplified by PCR from the genome. The DNA regions amplified by PCR are shown in dark blue; in light blue are the 5' and 3' *cfh3*$^+$ non-coding regions recovered from the genome; the ORF is shown in green.

**2.** A 1-kb-DNA fragment, located about 1 kb downstream of the *cfh3*$^+$ stop codon was PCR-amplified from the genome using oligos cfh3Hind (5'-ttc gtt att AAG CTT ccc taa ccc cag tat aaa aaa-3') and cfh3RI (5'-gaa gaa aca GAA TTC gaa ttt tca cgc ata gccgcg-3') This fragment was cloned into the *Hin*dIII and *Eco*RI sites in the pAU+5'cfh3 plasmid, giving rise to the pAU+5'+3'*cfh3*$^+$ plasmid.

**3.** The pAU+5'+3'*cfh3*$^+$ plasmid was digested with *Xho*I and *Hin*dIII and introduced in the HVP30 strain (see table 3 in the Apendix) by transformation. Transformants were selected on EMM-ura plates.

**4.** Plasmids were recovered from several independent yeast transformants and transformed into *E. coli*. The recovered plasmids were confirmed by restriction analysis. The plasmids that presented the correct pattern of DNA fragments were saved for future work. They were named pAU+*cfh3*$^+$.

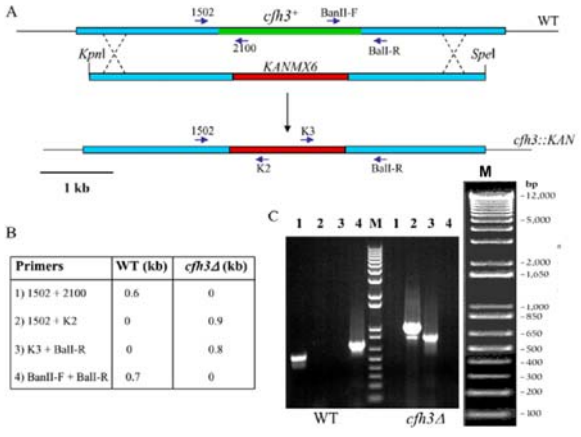
#### 3.3.2. Construction of a *cfh3::KAN* deletion cassette.

To study the effects of the absence of the *cfh3*$^+$ gene in the cells, we constructed a deletion cassette in which the *cfh3*$^+$ ORF was substituted by the KanMX6 gene (Bähler, 1998), which confers resistance to kanamycin in *E. coli* and resistance to geneticin (G418) in yeast. The construction was performed as follows:

**1.** The 5' and 3' *cfh3*$^+$ flanking sequences were cloned from the pAU+5'+3'*cfh3*$^+$ plasmid into the KS+ vector as a *Kpn*I/*Eco*RI fragment.

**2.** The KanMX6 was cloned in-between the yeast DNA sequences as a *Sal*I/*Hin*dIII DNA fragment. The new plasmid was termed KS+*cfh3::KAN*

In order to get null *cfh3* mutants the KS+*cfh3::KAN* plasmid was digested with *Kpn*I and *Spe*I. The product of the digestion was used to transform the HVP30 strain (see table 3 in the Appendix). Homologous recombination between the 5' and 3' *cfh3*$^+$ non-coding regions in the plasmid and the chromosome allows the replacement of the *cfh3*$^+$ ORF by the KANMX6 gene (see figure 46 A). Potential positive clones were recovered from the YES+G418 plates. In order to select those transformants in which the DNA fragment was stably integrated in the genome, replicas were done for 5 consecutive days on YES medium. Finally, the colonies were replica-plated on YES+G418 plates. Those clones that grew on YES+G418 plates were selected to proceed with the analysis




| Primers | WT (kb) | *cfh3Δ* (kb) |
|---|---|---|
| 1) 1502 + 2100 | 0.6 | 0 |
| 2) 1502 + K2 | 0 | 0.9 |
| 3) K3 + BalI-R | 0 | 0.8 |
| 4) BanII-F + BalI-R | 0.7 | 0 |

**Figure 46. Construction of a *cfh3Δ* null mutant**. The *cfh3*$^+$ ORF was replaced by the KANMX6 gene giving rise to the *cfh3::KAN* allele. (A) Schematic representation of the wild-type (WT) or *cfh3::KAN* alleles. Blue arrows represent the relative position of the oligonucleotides used to confirm that the *cfh3*$^+$ ORF was replaced by the KANMX6 module. (B) Expected size of the DNA fragments amplified by PCR with the indicated oligonucleotides. (C) DNA fragments amplified by PCR using the indicated sets of oligonucleotides (1 to 4, according to B) and genomic DNA from a WT (left lanes) strain and a putative *cfh3Δ* null mutant (right lanes). M: molecular weight marker 1 kb Plus DNA ladder. The right panel shows an enlarged view of the DNA fragments in this marker and their corresponding size.

Finally, to check that the deletion cassette was integrated properly at the *cfh3*$^+$ *locus* total DNA was recovered from the WT and some transformants and it was used to perform PCR reactions with oligos that were internal and external to the deletion cassette (see figure 46 B). The result obtained from the WT and a selected clone is shown in figures 46 C. This result showed that the *cfh3::KAN* null mutants are viable. This new strain was named HVP630. Later, new strains were constructed using this procedure, or by genetic crosses using HVP630 as a parental, depending on the needs.

### 3.3.3. Construction of overexpression plasmids

A pREP3X+*cfh3*$^+$ plasmid was constructed to determine the effect of overexpressing the *cfh3*$^+$ gene in the cells. The construction was as follows:

**1.** The *cfh3*$^+$ gene was subcloned, as a *Bgl*II/*Bgl*II DNA fragment into the *Bam*HI site in the polylinker of the KS+ vector.

**2.** The KS+*cfh3*$^+$(BglII) plasmid was used to obtain ssDNA using the protocol described in (Kunkel *et al.*, 1987).

**3.** Site-directed mutagenesis was used to introduce an *Xho*I site just upstream of the initial ATG codon and a *Not*I site just downstream of the ATG. Oligo cfh3XhATGNt (5'-cc caa ggg gct tct att tcg cat aga aga tgc tga atc GCG GCC GCC **cat** CTC GAG taa tgg act ttt tag gag aat agt cag att agc aaa aga aaa ctc g-3') was used for this purpose. This plasmid was named KS+*cfh3*$^+$(BglII)XAN.

**4.** An *Xho*I/*Sac*I DNA fragment, containing the *cfh3*$^+$ ORF and terminator sequences was cloned into the *Xho*I and *Sal*I sites of the pREP3X vector. This plasmid was used to analyze the phenotype produced by the overexpression of the *cfh3*$^+$ genes in the strains of interest.

**5.** In order to study the localization of the Cfh3 protein when the gene was overexpressed, the GFP protein was cloned as a *Not*I/*Not*I DNA fragment in the *Not*I site located downstream of the ATG in the pREP3X+*cfh3*$^+$ plasmid.

### 3.3.4. Construction of tagged versions of the *cfh3*$^+$ gene

Different tags were introduced in the *cfh3*$^+$ gene, just downstream of the initiation codon, to fulfill different purposes. Several steps were required to get these constructs:

**1.** A *Kpn*I/*Kpn*I DNA fragment, carrying the *cfh3*$^+$ 5' sequences and 1.4 kb from the ORF, was subcloned into a modified version of the KS vector, lacking the *Not*I site in the polylinker.

**2.** ssDNA obtained form the KS(-Not)+cfh3 (Kpn/Kpn) plasmid and oligo cfh3XhATGNt (see above) were used to introduce an *Xho*I site just upstream of the initial ATG codon and a *Not*I site just downstream of the ATG.

**3.** Then, the green fluorescent protein, the Tomato red protein or the HA epitope were introduced as *Not*I/*Not*I DNA fragments. Proper orientation was determined by restriction analysis.

**4.** The full-length tagged versions of the gene were cloned in the integrative pJK148 vector. To do so, triple ligations were performed using the vector digested with *Kpn*I and *Sac*I, a *Kpn*I/*Bam*HI (in the case of the GFP or the To-RFP constructs) or a *Kpn*I/*Nde*I (for the HA construct) DNA fragment from the plasmid mentioned above and a *Bam*HI (or *Nde*I, depending on the case)/*Sac*I DNA fragment, containing the terminal part of the genes, from the pAU+*cfh3*$^+$ plasmid.

**5.** In order to integrate these plasmids at the *leu1*$^+$ *locus* in the yeast strains of interest, the plasmids were linearized by digestion with *Tth*111I.

### 3.3.5. Construction of mutated versions of the *cfh3*$^+$ gene

To construct mutated versions of the *cfh3*$^+$ gene in which several or all the SEL1 domains were missing, site directed mutagenesis was used to introduce *Pma*CI restriction sites 10 aminoacids upstream or downstream of the domains of interest, which were eliminated by digestion with this enzyme and plasmid relegation. Oligos Cfh3SEL(1-2)5' (5'-cgaaatacattgctttCACGTGagcattctctaactc-3') and Cfh3SEL(1-2)3' (5'-ccaacagcatgcactCACGTGgtcattctctgaaac-3'), Cfh3SEL(3-5)5' (5'-gacagctcccattcgCACGTGTAATTGAGCCGATGA-3') and Cfh3SEL(3-5)3' (5'-ctcaattgcttcactCACGTGatgtcctgctttagc-3'), or Cfh3SEL(1-2)5' and Cfh3SEL(3-5)3' and ssDNA from the KS+*cfh3*$^+$(BglII)XAN plasmid were used to perform the mutagenesis.

In order to analyze the relevance of the prenylation site in the Cfh3 protein, site-directed mutagenesis was used to substitute its coding sequence by the AAGCTT sequence (corresponding to a *Hin*dIII site) from the gene. Oligo Cfh3-CIIS (5'-ggg agc aaa aat tgc caa ctt aAA GCT Tgt tat gtt tta taa gaa act ttt tag atg tct ttt tgg g-3') and ssDNA from the KS+*cfh3*$^+$ (BglII)XAN plasmid were used. In this way, the CIIS sequence was changed to KL. The accuracy of all the constructions was assessed by DNA sequencing.

## 4. Protein analysis

General techniques for protein analysis were performed according to (Harlow, 1988) with some variations.

### 4.1. Protein extraction

Cells from a minimum of 30 milliliters of a culture that had an $O.D._{600}$ = 0.5 - 1.0 were collected, washed with cold water, and passed to a 1.5 ml eppendorf-type tube. 5 µl of extraction buffer (50 mM HCl-Tris, pH 7.2; 350 mM NaCl; 50 mM EDTA; 1 mM PMSF; 1 µg/ml Aprotinin, Leupeptin, and Pepstatin, unless stated otherwise) per O.D. were added to the samples. Ice-cold Ballotini (0.45 nm diameter; *'Glasperlen', Braun Biotech International*) were added to the samples and then cells were broken in a "Fast Prep" FP120 (*Savant; Bio101*) 3 times for 15''at a speed of 5.5 and at 4ºC. Cell extracts were transferred into a clean cold eppendorf by pricking the tube with a hot needle, putting it into the clean tube and centrifuging it at 3000 rpm for 1 minute in a cold microfuge. Supernatants were transferred to a clean tube

and protein was estimated by using the *Biorad protein assay kit* (Bradford method). Estimation of protein concentration was done using duplicates from 1:10 dilutions from the samples, and BSA as standard (0-5-10-15-20 µl from a 1 mg/ml solution). For Western blotting analysis, the volume of sample containing 100 µg of protein was combined with 2X sample buffer (please see composition in the appendix) and heated for 4 minutes at 100ºC.

### 4.2. Western blotting analysis

Protein electrophoresis and blotting were performed following the techniques described in (Laemli, 1970, Harlow, 1988). Proteins were loaded into polyacrilamide gels at a concentration of 6.5%, since the bands of interest were of high molecular weight, with 1% SDS in the buffer (Laemmli buffer; appendix). The electrophoresis was performed for about 1 hour at 150 V. The proteins were then transferred to PVDF membranes, InmobilonTM-P (Millipore), using Tris-Glicine transfer buffer (appendix) and the "*Mini Trans Blot*" (BioRad) system for 3.5 hours in the cold at 100 V. In order to avoid unspecific union of the antibody, the membrane was blocked with 10% skimmed milk (Sveltesse-Nestlé) dissolved in TBST -TBS (appendix) containing 0.25% Tween-20 (Sigma)- for 1 hour at room temperature. Next, the membrane was incubated with the primary antibody (monoclonal anti-HA, 12C5A, Roche; monoclonal anti-GFP, JL-8, Nucliber, or with polyclonal anti-phospho-p44/42 MAP kinase, Thr202/Tyr204, Cell Signalling Technology) diluted in the same blocking solution (1:4000, 1:500, and 1:2500 dilution, respectively) overnight at 4ºC. Afterwards, the membranes were washed 2 times with TBST for 10 minutes, and were incubated with the secondary antibody (antimouse or antirabit, Amesham; 1:10000 dilution in the blocking solution) at room temperature for 1 hour. Finally, 2 washings with TBST for 10 minutes were preformed.

ECL or ECL Advanced kits (Amersham), based on the detection of the activity of the peroxidase enzyme linked to the secondary antibody, were used to develop the Western blots.

### 4.3. Co-immunoprecipitation

Immunoprecipitation is one of the most widely used methods for antigen detection and purification. An important characteristic of immunoprecipitation reactions is their potential to deliver not only the target protein but also other macromolecules that interact with the target (in this case is called co-immunoprecipitation). In these methods an antibody is allowed to form an immune complex with the specific target antigen present in a sample, such as a cell lysate. The immune complex is then captured on a solid support, usually sepharose beads, to which either Protein A or Protein G has been immobilized (Protein A or G binds to the antibody). The process of capturing this complex from the solution is referred to as precipitation. The proteins that are not "precipitated" by the immobilized Protein A or G support are washed away. Finally, components of the bound immune complex (both antigen and antibody) are eluted from the support and analyzed by SDS-PAGE (gel electrophoresis), often followed by Western blotting detection to verify the identity of the antigen.

Co-immunoprecipitation is a widely-used technique for detecting protein-protein interactions. Many protein-protein associations that exist within the cell remain intact when a cell is lysed under nondenaturing conditions. Thus, if protein "X" is immunoprecipitated, then protein "Y", which stably associated with "X", may also precipitate. Co-immunoprecipitation is conducted in essentially the same manner as an immunoprecipitation. However, in a co-immunoprecipitation the target antigen precipitated by the antibody "co-precipitates" a binding partner/ protein complex from a lysate, i.e., the interacting protein is bound to the target antigen, which becomes bound by the antibody that becomes captured on the Protein A or G gel support. Co-immunoprecipitation is most commonly used to test whether two proteins of interest are associated (directly or indirectly) *in vivo*, but it can also be used to identify interacting partners of a target protein.

For our experiments, cells from a minimum of 150 milliliters of a culture that had an $O.D._{600}$ = 0.5 - 1.0 were collected by centrifugation, washed with 1 ml ice-cold water, transferred to 1.5 ml eppendorf tubes,

and 5 µl of extraction buffer (50 mM HCl-Tris, pH 7.5; 200 mM NaCl; 5 mM EDTA; 0.5% IGEPAL CA-630; 1 mM PMSF; 1 µg/ml Aprotinin, Leupeptin, and Pepstatin) per O.D. were added to the samples. Then, the protein extraction was performed as it has been described above (Section 4.1). Next, 5 mg protein from each extract was put in cold tubes and the volume of all tubes was taken to 330 µl using extraction buffer. Then, 30 µl from every sample were transferred into a new cold tube, and 30 µl 2XSB were added into the tubes. These samples were used as "Total Extracts" samples. 300 µl of IP (immunoprecipitation) buffer (50 mM HCl-Tris, pH 7.5; 200 mM NaCl; 5 mM EDTA; 2% Triton X-100; 1 mM PMSF; 1 µg/ml Aprotinin, Leupeptin, and Pepstatin) were added to the rest of extracts. Next, 2.5 µl Rabbit anti-GFP antibody (Invitrogen, anti-GFP, serum, catalogue number A6455) were added to the samples, which were incubated for 2 hours at 4ºC in a tube rotator. Then, 50 µl of Protein A-Sepharose CL-4B beads (Pharmacia Biotech; 0.1 g/ml in IP buffer) were added to the samples, and all the mixture was incubated in a tube rotator at 4ºC over night. Next day, the beads were washed 3 times using the same IP buffer and 1 time with PBS (Phosphate Buffered Saline). Soft and short centrifugations (1000 × g for 1 minute) were used to precipitate the beads. After the last wash all the liquid was removed from the samples, 50 µl 2X sample buffer (appendix) were added to the beads and the beads were incubated at 100ºC for 4 minutes in order to release the antigen. Finally, a short centrifugation was performed at high speed (13,200 rpm) and the supernatants were loaded into the acrylamide gel. Protein electrophoresis from the "total extracts" (15 µl in each of 2 gels) and the immunoprecipitated samples (15 µl in a gel, to be developed using anti GFP antibody, and 35 µl in a second gel to be developed using anti-HA antibody), as well as transferring, were performed following the techniques described above (Section 4.2).

### 4.4. Rhotekin binding Analysis

*E. coli* DH5α cells were transformed with the pGEX-C21RBD plasmid that carries a sequence coding for the C21 peptide (corresponding to the Rho Binding Domain) in the Rhotekin protein fused to the GST sequence (Reid *et al.*, 1996). Then, the transformed bacteria were incubated at 28ºC in 400 ml LB media supplemented with the antibiotic (Ampicillin 50 µg/ml) until they reached an $O.D._{600}$ = 0.6 – 0.8. Then, IPTG was added at a final concentration of 0.5 mM to induce the expression of the gene that codes the fusion protein, and cells were incubated for three additional hours at 28ºC. Afterwards, the cells were collected by centrifugation, and resuspended in 20 ml of cold PBS (appendix) supplemented with different protease inhibitors (1 mM PMSF; 2 µg/ml Aprotinin, Leupeptin, and Pepstatin), and lisozyme at a final concentration of 2 mg/ml. After 30 minutes of incubation into the ice, the cells were sonicated four times at a power of 14 µm of amplitude wave during 1 minute. Triton-X100 at a final concentration of 1% was added to the cells, which were centrifuged at 7800 × g for 1 hour at low temperature. Next, the supernatant was taken and incubated at 4ºC with sepharose beads bound to glutathione (Pharmacia; prepared in PBS). Then, the glutathione-sepharose beads, which are now united with the fusion protein GST-RBD, were washed three times with 40 ml PBS, and centrifuged at low speed (1000×g). Finally, they were resuspended in lysis B solution (appendix), supplemented with protease inhibitors. The amount of protein was quantified in a 12.5% polyacrilamide gel comparing the sample with different amounts of bovine seroalbumine.

In order to perform the rhotekin binding analysis, the corresponding strains were inoculated in YES medium and incubated overnight at 28ºC. Next morning, the strains were refreshed in 50 ml media, so that at the time of starting the experiment the $O.D._{600}$ did not go above a value of 1. Then, the cells were collected, washed with 1 ml cold EDTA, and passed to a 1.5 ml eppendorf. 5µl of cold Lysis Buffer B (0.5% IGEPAL; 20 mM NaCl; 10% Glycerol; 2mM MgCl2; 0.1 mM DTT; 50 mM Tris HCl pH 7.6; 1 mM PMSF; 1µg/ml Aprotinin, Leupeptin, and Pepstatin) per O.D. were added to the samples. Breaking the cells and extraction of the proteins were done as explained in Section 4.1. Next, the extracted proteins were centrifuged at 5000 rpm for 10 minutes in the refrigerated centrifuge, and the supernatants were transferred into cold clean tubes. Afterwards, the amounts of proteins were estimated as it has been explained (Section 4.1). Then, 2 mg protein from each extract was put in a cold tube, and the volume was increased to 500 µl with cold Ly-

sis Buffer B supplemented with inhibitors of proteases. "Total Extracts" samples (At a concentration of 2 µg/µl) were taken as described previously (Section 4.3). To the rest of the extracts, 30 µl GST-RBD Sepharose beads in Lysis Buffer B, already bound to rhotekin (with a concentration of 0.3 mg/ml Rhotekin) were added, and incubated over night at 4ºC. Next morning, the beads were washed 4 times with 1 ml of Lysis Buffer B supplemented with protease inhibitors as described previously (Section 4.3). Afterwards, 30 µl 2X sample buffer (appendix) were added to the beads that were incubated at 100ºC for 4 minutes in order to release the antigen. Finally, a short centrifugation was performed at high speed and the supernatants were loaded in a 12% polyacrilamide gel. Protein electrophoresis and transferring were performed following the techniques described previously (Section 4.2).

## 5. Analysis of Spm1/Pmk1 activation

In order to analyze activation of the Spm1 pathway under different conditions, the strains of interest were inoculated in the corresponding media and grown at 28ºC overnight. Next morning, the strains were refreshed and incubated at 28ºC so at the time of starting the experiment we had a 60 ml-culture at an $O.D._{600}$ = 0.5. Then, 30 ml from the culture were transferred to a flask containing solid KCl, so it was at a final concentration of 0.6 M (1.34 g KCl). Both flasks were incubated at 28ºC for an additional 15 minutes. Afterward, the cells were filtered, and the filter was transferred to a tube containing 1 ml cold PBS (all work was done into the ice). Next, the cells were collected by giving a short spin (4 seconds at 3000 rpm) in a refrigerated centrifuge, and the liquid and filter were discarded. The cells were resuspended into 150 µl cold Lysis Buffer (50mM HCl Tris pH 8; 150 mM NaCl, 10% Glycerol, 0.1 % IGEPAL CA-630) with protease inhibitors, and cold Ballotini was add to the mixture. Breaking the cells, extraction and estimation of the protein concentration were done as explained previously (Section 4.1) with the exception that the centrifugation to remove the cell debris from the lysate was performed for 10 minutes at 13000 rpm. Then, supernatants were transferred to clean tubes. 800 µg of protein from each sample was taken to the same final volume, and 60 µl Nickel-NTA beads (Novagen; Ni-NTA His Bind Resin Beads), previously equilibrated with PBS, were added and incubated at 4ºC for 2 hours. To equilibrate the Ni-NTA beads they were centrifuged, washed 3 times with 1 ml PBS and resuspended in the original volume of PBS.

Next, the beads were washed 3 times with 0.5 ml of cold 1 M Tris-HCl pH 8 by Centrifugation at 3000 rpm for 1 minute. After eliminating all liquid, 30 µl 2XSB (appendix) were added into the beads, and the beads were incubated at 100ºC for 4 minutes in order to release the antigen. Finally, a short centrifugation was performed at maximum speed and the supernatants were loaded into a 10% polyacrilamide gel. Protein electrophoresis and transferring were performed following the techniques described above (Section 4.2). Anti-Phospho p42/44 antibody (1:2500 dilutions, Section 4.2) and anti-Rabbit (1:10000 dilutions) were used as primary and secondary antibodies, respectively, for developing the Western blot. Afterwards, in order to detect the total amount of Spm1 protein loaded into the gel, stripping of the membrane was preformed by washing it with the following solutions: 100 mM Glycine at pH 2.5 for 15'; PBS 3.5 M MgCl2 for 15', the membrane gets transparent in this stage; water until the membrane is white again; 1% SDS for 15'; and TBST for15'. Then, the membrane was incubated in 10% milk in TBST for 1 hour. Finally, the membrane was developed again using anti-HA antibody (1:4000 dilution) and anti-mouse antibody (1:10000 dilutions) as primary and secondary antibodies, respectively.

## 6. Cell wall analysis

### 6.1 Sensitivity to digestion by glucanases

The strains of interest were inoculated in the corresponding media and incubated overnight at 25ºC. Next morning they were refreshed and incubated at 25ºC so that at the time of the experiment they were at an $O.D._{600}$ = 1. Then, the amount of cells needed to have 2 ml of an $O.D._{600}$ = 2 were harvested and washed with 50 mM citrate/phosphate buffer pH 5.6 (see the composition in the appendix). Next, they were resuspended in 2 ml 50 mM citrate/phosphate buffer supplemented with different amounts of glucanases

(Zymolyase 100T or Novozyme 234) and incubated for 3 hours at 28ºC in a tube rotator. The $O.D._{600}$ was taken every half an hour during the incubation time, and the data were analyzed. Zymolyase-100T mainly degrades β(1,3)glucan and galactomannan, and Novozyme 234 is a crude extract able to degrade completely the *S. pombe* cell wall. Thus, cell wall-related mutants may show different sensitivities to Zymolyase and to Novozyme, depending on the importance of the α-glucan in maintaining cell integrity in this mutant (Perez & Ribas, 2004).

### 6.2 Fractioning

Exponentially-growing cultures from the strains of interest were diluted with media (YES or YES supplemented with KCl) without glucose to $O.D._{600}$ = 0.5 in a total volume 10 ml. Then, 5 µCi (25 µl of the actual $^{14}$C-Glucose) into each sample were added. The cultures were incubated for 4-6 hours (1 generation time) in the water bath shaker at 28ºC. Next, the cells were harvested by centrifuging at 3000 rpm for 5 minutes, transferred to tubes for FASTPREP (Safe lock eppendorf), and washed 2 times with 1 ml of 1 mM EDTA by centrifuging at 12000 rpm for 2 minutes at 4ºC (the tubes were emptied just inverting them in a container that used for liquid radioactive residues). Afterwards, the cells were resuspended in a final volume of 1 ml 1 mM EDTA, and 10 µl of these suspensions were taken and transferred to eppendorfs containing 10% TCA (taking samples was done in duplicate for each sample), and the samples were saved at 4ºC for next step as Aliquot 1 (Set "A", total incorporation).

The rest of the cells were centrifuged at 12000 rpm for 2 minutes at 4ºC, and supernatants were removed by inverting the tubes. Then, glass beads (Ballotini) were added to the samples (From this point, the experiment was done working into the ice). Next, cells were broken using the FASTPREP in the cold room (2 times, 20 second at speed of 5.5), and 500 µl 1 mM EDTA were added into the broken cells. Afterwards, cell extracts were recovered by punching the bottom of the tubes with a hot needle, setting the tubes into new tubes, and centrifuging them for 30 second at 3000 rpm at 4ºC. Samples were taken to a final volume of 1ml by adding 1 mM EDTA and they were resuspended by vortexing. Finally, 10 µl of these suspensions were taken and added to eppendorfs containing 10% TCA (duplicates were taken from each sample), and the samples were saved at 4ºC for next step as Aliquot 2 (Set "B", incomplete total incorporation).

The rest of the samples were centrifuged at 1000 × g for 10 minutes at 4ºC and supernatants were discarded. Then, they were washed 3 times with 1ml 2M NaCl (By centrifuging at 1000 × g for 5 minutes) and 1 time with 1 ml EDTA 1 mM. Afterwards, the cells were resuspended in a final volume of 1ml of 1mM EDTA, and 20 µl of these suspensions were taken and added to the eppendorfs containing 10% TCA (duplicates for each sample), and the samples were saved at 4ºC for next step as Aliquot 3 (Set "C", total walls).

The rest of samples were heated for 5 minutes at 95ºC and centrifuged at 1000 × g for 10 minutes at 4ºC. Next, they were resuspended in a final volume of 500 µl 1mM EDTA. Afterwards, the following reaction mixtures were prepared in total volume of 200 µl as follow:

Control ("C"): 50 µl of the walls supplemented with 50 µl citrate/phosphate buffer (pH 5.6, 50 mM) and 100 µl water.

Zymolyase-1 ("Z1"): 100 µl walls supplemented with 50 µl citrate/phosphate buffer (pH 5.6, 50 mM), 30 µl water and 20 µl Zymolyase 100T (10 mg/ml water).

Zymolyase-2 ("Z2"): 100 µl walls supplemented with 50 µl citrate/phosphate buffer (pH 5.6, 50mM), 40 ul water and 10 µl Zymolyase 100T (10 mg/ml water).

Glucanase-1 ("Q1"): 100 µl walls supplemented with 95 µl 50 mM Tris buffer (pH 7.5) and 5 µl (100 u) of Quantazyme

Glucanase-2 ("Q2"): 100 µl walls supplemented with 97 µl 50 mM Tris buffer (pH 7.5) and 3 µl (100 u) of Quantazyme.

Next, the above reaction mixtures were incu-

bated at 28ºC for about 36-40 hours and then, they were centrifuged for 3 minutes at maximum speed. Afterwards, 50 µl of the supernatant of all samples were transferred to scintillation vials containing 2.5 ml scintillation solution (OptiPhase "Hi Safe", Wallac), and were directly counted in the scintillation counter (Wallac 1409) as Aliquot 4.

1 ml 10% TCA was added to the rest of reaction mixtures, and then the pellets were resuspended. These samples were used as Aliquot 5.

All TCA precipitates (Aliquot 1, 2, 3, and 5) were filtered with Watman (Watman GF/C) filters and washed 2 times with 2.5 ml 10% TCA and then with 3 - 4 ml 96% ethanol. Then the filters were dried in the scintillation tubes and 2.5 ml scintillation solution was added to the tubes. Finally, all samples were counted in the scintillation counter.

Note that the precipitate from the incubation with Zymolyase 100T corresponds to the α-glucan and the supernatant from the incubation with Quantazyme corresponds to β(1,3)glucan. The difference between the precipitates of Quantazyme and Zymolyase 100T correspond to galactomannan plus β(1,6)glucan.

Calculation was done as follow: first, the mean value was calculated from the duplicated samples and then the following calculations were done for each Aliquot.

Aliquot 1: 10 µl × 100 = total incorporation
Aliquot 2: 10 µl × 100 = incomplete total incorporation
Aliquot 3: 20 µl × 50 = Total walls
Aliquot 4: Supernatant
*Control: spontaneous degradation
*Zymolyase: β-glucan + Manan: 50 µl × 4 × 5
*Glucanase: β-glucan: 50 µl × 4 x 5
Aliquot 5: Precipitate
*Control: Total walls 50 µl × 10
*Zymolyase: α-glucan 100 µl × 5
*Glucanase: α-glucan + Manan 100 µl × 5

The results presented in this work are the averages of the values obtained in 5 independent experiments, with duplicated samples.

## 7. Glucan synthase assays

All work was performed at 0–4ºC in order to preserve the enzymatic activities. Strains were inoculated in YES medium and incubated at 25ºC overnight. Next day, cultures were refreshed and incubated at 25ºC for 4 hours so that at the time of experiment, we had 100 ml cultures at an $O.D._{600}$ = 1. Then, every culture was split into 2 flasks, 50 ml YES medium were added to one set and 50 ml YES medium supplemented with 2 M KCl to the other set (in order to check the activity of enzyme under stress conditions). Cultures were incubated at 25ºC for 20 minutes. Afterwards, cells were harvested by centrifugation at 3000 rpm for 5 minutes, washed with 10 ml of Washing Buffer (appendix), and transferred to an eppendorf with 1 ml washing buffer. Next, cells were given a short spin and the supernatants were discarded. Then, 120 µl of washing buffer, 1 µl GTP-γ-S (10 mM), and cold Ballotini were added to the cells. Breaking the cells and extraction of the proteins were done as explained (Section 4.1). Afterwards, the extracts were transferred to 15-ml cold tubes, diluted to 15 ml with cold washing buffer, vortexed for a few seconds, and centrifuged at 3000 rpm for 5 minutes in a cold centrifuge to eliminate the cell walls and glass beads. Next, the supernatants were transferred to clean 30-ml tubes, centrifuged at 4ºC for 30 minutes at 18000 rpm in a Beckman JA-20 rotor to collect the membranes. At the end of centrifugation, all the supernatants were eliminated and the sediments were resuspended in 100 µl Resuspension Buffer (appendix) by using glass rods and vortex in two steps (each step 50 µl Resuspension buffer was added). Protein was estimated as it has been explained (Section 4.1). Then, the reaction mixture was set up with [$^{14}$C] UDP-D-glucose as a substrate.

Reaction mixtures containing the following components were prepared:

- 75 mM HCl-Tris pH 8.
- 5 mM [$^{14}$C]UDP-D glucose ($2\times10^5$ cpm/µmol).
- 0.75% Bovine Seroalbumine.
- 2.125 mM EDTA.

All these components were taken to a final volume of 40 µl, out of which 5 µl corresponded to the membrane extract. Then, reaction was incubated at 30ºC for 30 minutes. After that time, the reaction was stopped by adding 2.5 ml of 10% TCA and incubated at 4ºC for a minimum of 30 minutes. Next, samples were filtered and counted as explained previously (Section 6.2). The results presented in this work are the averages of the values obtained in at least 3 independent experiments with duplicates. The enzymatic unit is defined as the amount of enzyme that catalyzes the incorporation of 1µmol of substrate (UDP-D-glucose) per minute at 30ºC.

## 8. Microscopy

A Zeiss microscope, model Axioskop 2 extra, equipped with systems of halogenous illumination and equipment of digital photography, was used in order to carry out different microscopic techniques such as DIC, phase contrast, and florescence microscopy for routine work.

### 8.1. Fluorescence microscopy

The observation of samples with low emission of fluorescence and the acquisition of quality photographic images was performed in a DM RXA Leica microscope by illuminating the samples with an epifluorescence system using a mercury lamp of 100 w (EBQ100). This microscope was equipped, in addition, with a system of halogenous illumination as well as a system of filters for microscopy of phase contrast and differential interference contrast (DIC). A digital camera Leica Qsensys with its associated informatics program, Qfish v 2.3, was used for taking images. For the observation of samples with fluorescence, the suitable filters were used to let the UV light of a certain wavelength pass, according to the spectrum of excitation-emission of the fluorochromes or fluorescent proteins that were used. Finally, the obtained images were processed with the program Adobe Photoshop version 7.

#### 8.1.1. Staining with Hoechst 33258

In certain cases we were interested in the study of the localization of some GFP-fused proteins with respect to the septum and/or poles, or with respect to the stage of the cellular cycle in which cells were. In these cases, it was interesting to perform a cell wall and/or nuclear staining on living or fixed cells. To do this, Hoechst 33258 was used. Hoechst dye binds preferably to A/T-rich zones in the double chain of DNA. It is excited by ultraviolet light at around 350 nm, and emits blue/cyan fluorescent light at an emission maximum of 461 nm. The permeability of Hoechst 33342 is very high in the absence of fixation. However, we chose the Hoechst 33258 variant because it is very soluble and because, as a consequence of its lower permeability into the cells, it allows the observation of the cell wall by unspecific staining.

For the living cells staining, cells from 1 ml of a culture at an O. $D._{600}$ = 0.5 – 1 were harvested, washed with 1 ml of water, and resuspended in 20 µl of a 1/20 Hoechst 33258 dilution (prepared from a stock solution at 1 mg/ml in $H_2O$, so it is used at an 1.8 µ M final concentration). Cells were incubated for 5-10 minutes at room temperature, washed twice with $H_2O$, and observed under the microscope.

#### 8.1.2. Staining with Calcofluor

Calcofluor binds (1,3)β and (1,4)β polymers of the cell wall of yeasts, reason why it is used to detect anomalies in the wall or to determine the places of growth. Calcofluor white is a fluorochrome that in *S. cerevisiae* essentially dyes the zones of cell wall rich in chitin. In *S. pombe,* in spite of not having chitin, binds to the septa and the cell poles (places of active synthesis of cell wall) because it binds to lineal (1,3)β-glucan with some affinity. Calcofluor is excited by ultraviolet light at around 372 nm wavelength and emits blue fluorescent light. Calcofluor (Blancophor BBH, Bayer Corporation) was prepared in water at different concentrations depending on experiments (normally the stock solution was prepared at 20 or 10 mg/ml). Because it is not soluble at acidic pH, some drops of 10 N KOH were added to the solution to help its dissolution. Next, the solution was filter-sterilized and kept at 4ºC protected from the light.

In order to perform cell staining, cells from a 1ml-culture at $D.O_{600}$ = 1 were harvested by centrifugation at 3000 rpm for 1 minute, washed with 1 ml water, resuspended in a water solution with Calcofluor at different concentrations (normally at 0.1 mg/ml final concentration), and incubated for 5 minutes at room temperature. Finally, cells were washed and concentrated to observe them under the fluorescence microscope.

### 8.1.3. Actin staining (Rhodamine-Phalloidin)

Phalloidin, a toxin extracted from the fungus *Amanita phalloides*, binds F-actin (filaments of actin) with high affinity. Taking advantage of this property, it can be used conjugated to a fluorescent compound to detect actin in the cells. In our case, Phalloidin was conjugated with Rhodamine. Actin staining was done by following the indications and modifications performed by F. Chang from the initial protocol described by (Marks, 1985).

Cells growing in exponential phase (from a 5 ml-culture) were fixed in a tube rotator for 1 hour at room temperature by adding 0.75 ml 16% Formaldehyde (methanol-free Electron-Microscope grade, Poly-Science) plus 1.5 ml PEM buffer (appendix). Next, cells were washed 3 times with PEM buffer and, then, permeabilized for 30 seconds with the same buffer containing 1% Triton-X100 to allow the Rhodamine-Phalloidin to enter into the cell. Cells were washed with PEM buffer again, 0.5 µl from the cells were taken and 4 µl of Rhodamine-Phalloidin (Molecular Probes, resuspended in PEM buffer) were added. Finally, the samples were incubated in the dark for 45-60 minutes in a tube rotator and examined under florescence microscope.

### 8.1.4. Time-Lapse experiments

"Time-lapse" studies are performed to study the evolution of a cellular process by taking pictures under the microscope at regular time-points and comparing the images obtained. To do this, it is necessary to keep cells under optimal survival and growth conditions. It is also necessary to immobilize the cells on the microscope slides so images can be taken along time.

In our case, "Time-lapse" studies were performed taking pictures from the samples every 7 minutes. Samples were prepared in two ways; initially, the preparation of samples was performed in an eppendorf tube by mixing an equal volume of the liquid culture with the same culture medium prepared with 1% low melting point agarose, melted, and kept warm at 42ºC. The sample was then mixed by pipeting and 3 µl-aliquots were put on microscope slide. The sample was covered with a cover glass, without sealing the preparation, and observed along time. These observations were performed in a room at 28ºC. In a second protocol, cells from a 5 ml-culture were filtered, collected with a pipette pipette tip from the surface of the filter, and resuspended in 50 µl of the same medium. 5 µl from this sample were spread onto a solid medium previously placed into the cave of a microscope slide with cavity. Next steps were done as explained above.

### 8.1.5. Confocal microscopy

In order to observe cell structures such as the contractile ring in more detail, and/or to have a 3-dimensional composition from these structures, a spectral laser confocal microscope (Leica TCS-SL) was used. 20-30 cross-sectional images of all the cellular volume (z-stacks), with a thickness of 0.2 µm were obtained from the cells. These images were, then, processed by a program associated to the microscope, denominated Leica Confocal Software (LCS) to obtain the maximum or average orthogonal projection (according to the cases) of these sections. This program was also used to perform the reconstruction in 3 dimensions of the acquired images.

# Appendix

**Composition of the buffers frequently used in this work:**

### Laemmli Buffer 1X

| | |
|---|---|
| Tris-base | 3 g/l |
| Glycine | 14.4 g/l |
| SDS | 1 g/l |

### Transfer Buffer

| | |
|---|---|
| Tris-base | 3 g/l |
| Glycine | 14.4 g/l |
| Methanol | 10 % |

### 2X Sample Buffer

| | | |
|---|---|---|
| HCl-Tris | pH 6.8 | 100 mM |
| SDS | | 2 % |
| β-mercapto ethanol | | 290 mM |
| Glycerol | | 20% |
| Bromphenol blue | | .004% |

### TBS

| | |
|---|---|
| Tris-base | 2.5 g/l |
| NaCl | 9 g/l |

### PBS

| | |
|---|---|
| Na2HpO4 | 10 mM |
| KH2PO4 | 2 mM |
| NaCl | 137 mM |
| KCl | 2.7 mM |

### Lysis B solution

| | | |
|---|---|---|
| NP-40 | | 0,5 % |
| NaCl | | 20 mM |
| Glycerol | | 10 % |
| MgCl2 | | 2 mM |
| Ditriotreitol | | 0,1 mM |
| Tris-HCl | pH 7,6 | 50 mM |

### 50 mM citrate/phosphate buffer

| | |
|---|---|
| Na2HPO4 | 7.1 g/l |
| Citric acid | 11.5 g/l |
| PH | 5.6 |

### GS Washing Buffer

| | | |
|---|---|---|
| Tris | pH 8 | 50 mM |
| EDTA | | 1 mM |
| β-Mercaptoethanol | | 1 mM |

### GS Resuspention Buffer

| | | |
|---|---|---|
| Tris | pH 8 | 50 mM |
| EDTA | | 1 mM |
| β-Mercaptoethanol | | 1 mM |
| Glycerol | | 30% |

### PEM Buffer

| | |
|---|---|
| PIPES | 100 mM |
| EGTA | 1 mM |
| Mg2SO4 | 1 mM |
| pH | 6.9 |

## Composition of the culture media used in this work:

### *Schizosaccharomyces pombe* culture media:

* Used for the vegetative growth: YES Medium, Minimum Medium EMM (Edinburgh Minimal Medium)

** Used to induce the sporulation

### YES Medium*

| | | |
|---|---|---|
| Yeast extract | 5 g/l | 0.5% (w/v) |
| Glucose | 30 g/l | 3% (w/v) |
| Supplements: adenine, histidine, leucine, lysine, and uracil | 225mg/l | |

### SPA Medium**

| | | |
|---|---|---|
| Glucose | 10 g/l | 1% (w/v) |
| KH2PO4 | 1 g/l | 0.1% (w/v) |
| Vitamins | | 1 ml/l |

### Minimal Medium EMM

| | | |
|---|---|---|
| Potassium hydrogen phthalate | 3 g/l | 14.7 mM |
| Na2HPO4 | 2.2 g/l | 15.5 mM |
| NH4Cl | 5 g/l | 93.5 mM |
| Glucose | 2% (w/v) | 111 mM |
| Salts | | 20 ml/l (stock x 1000) |
| Vitamins | | 1ml/l (stock x 1000) |
| Minerals | | 0.1 ml/l (stock x 10000) |
| **Salts x 50** | | |
| MgCl2.6H2O | 52.5 g/l | 0.26M |
| CaCl2.2H2O | 0.735 mg/l | 4.99mM |
| KCl | 50 g/l | 0.67 M |
| Na2SO4 | 2 g/l | 14.1 mM |
| **Vitamins x 1000** | | |
| Pantothenic acid | 1 g/l | 4.20 mM |
| Nicotinic acid | 10 g/l | 81.2 mM |
| Inositol | 10 g/l | 55.5 mM |
| Biotin | 10 mg/l | 40.8 µM |
| **Minerals x 10,000** | | |
| Boris acid | 5 g/l | 80.9 mM |
| MnSO4 | 4 g/l | 23 mM |
| ZnSO4.7H2O | 4 g/l | 13.9 mM |
| FeCl2.6H2O | 2 g/l | 7.40 mM |
| Molybdic acid | 0.4 g/l | 2.47 mM |
| KI | 1 g/l | 6.02 mM |
| CuSO4.5H2O | 0.4 g/l | 1.60 mM |
| Citric acid | 10 g/l | 47.60 mM |

### *Escherichia coli* culture media:

### LB Medium

| | | |
|---|---|---|
| Bactotryptone | 10 g/l | 1% (w/v) |
| Yeast extract | 5 g/l | 0.5% (w/v) |
| NaCl | 10 g/l | 1% (w/v) |

### 2xYT

| | | |
|---|---|---|
| Bactotryptone | 16 g/l | 1.6% (w/v) |
| Yeast extract | 10 g/l | 1% (w/v) |
| NaCl | 10 g/l | 1% (w/v) |

**Table 3: STRAINS USED IN THIS STUDY**

| STRAIN | GENOTYPE | ORIGEN |
|---|---|---|
| | ***Schizosaccharomyces pombe*** | |
| HVP 30 | *leu1-32 his3-Δ1 ura4Δ18 ade6 h⁻* | Lab. stock |
| HVP 162 | *leu1-32 his3-Δ1 ade6 h⁻* | Lab. stock |
| HVP 280 | *chs2::ura4⁺ leu1-32 ura4-Δ18 his3-Δ1 ade6 h⁻* | Lab. stock |
| HVP 458 | *leu1-32 h⁻* | P. Nurse |
| HVP 513 | *spn3::ura4⁺ leu1-32 ade6 h⁺* | Jurg Bähler |
| HVP 514 | *spn4::ura4⁺ leu1-32 ade6 h⁻* | Jurg Bähler |
| HVP 530 | *cdc11-119 leu1-32 h-* | Lab. stock |
| HVP 531 | *cdc14-118 leu1-32 h-* | Lab. stock |
| HVP 532 | *cdc15-140 leu1-32 h⁻* | Lab. stock |
| HVP 533 | *cdc16-116 leu1-32 h⁺* | Lab. stock |
| HVP 607 | *imp2::ura4⁺ leu1-32 ade6h⁻* | Shelly Sazer |
| HVP 630 | *cfh3::KAN leu1-32 his3Δ1 ura4-Δ18 ade6 h⁻* | This work |
| HVP 631 | *cfh3::KAN leu1-32 his3Δ1 ura4-Δ18 ade6 h⁻* | This work |
| HVP 652 | *cdc4-8 leu1-32 h⁺* | P. Nurse |
| HVP 687 | *chs4::ura4⁺ cfh3::KAN leu1-32 his3-Δ1 ade6 h⁻* | This work |
| HVP 724 | *cfh1::ura4⁺ cfh2::KAN cfh3::KAN cfh4::KAN leu1-32 his3Δ1 ade6 h⁻* | This work |
| HVP 726 | *cfh1::ura4⁺ cfh2::KAN cfh3::KAN leu1-32 his3-Δ1 ade6 h⁻* | This work |
| HVP 775 | *cfh2::KAN cfh3::KAN leu1-32 his3Δ1 ura4-Δ18 ade6 h⁻* | This work |
| HVP 779 | *cfh3::KAN cfh4::KAN leu1-32 ura4-Δ18 his3-Δ1 ade6 h⁻* | This work |
| HVP 786 | *myo2E1 myo3::ADE2 leu1-32 ura4-Δ18 h⁺* | Lab. stock |
| HVP 789 | *cfh2::KAN cfh3::KAN cfh4::KAN leu1-32 ura4-Δ18 ade6 h⁻* | This work |
| HVP 819 | *cps8-188 leu1-32 h⁺* | P. Perez |
| HVP 994 | *cdc15-GFP ::ura4⁺ leu1-32 ura4-Δ18 h⁻* | S. Moreno |
| HVP 999 | *eng1-GFP::KAN leu1-32 ura4-Δ18 ade6 h⁻* | F. del Rey |
| HVP 1000 | *agn1-GFP::KAN leu1-32 ura4-Δ18 ade6 h⁻* | F. del Rey |
| HVP 1051 | *cdc4-GFP::ura4⁺ leu1-32 ade6 h⁻* | M. Balasubramanian |
| HVP1053 | *rlc1-GFP::KAN leu1-32 ura4-Δ18 h⁻* | P. Perez |
| HVP 1098 | *hht2-GFP::ura4⁺ leu1-32 ade6 h⁺* | M. Balasubramanian |
| HVP 1139 | *cps1-191 leu1-32 h⁻* | J.C. Ribas |
| HVP 1213 | *spn3-GFP::KAN leu1-32 ura4-Δ18 ade6 h⁺* | M. Balasubramanian |
| HVP 1244 | *rlc1::KAN leu1-32 ura4-Δ18 ade6 h⁻* | V. Simanis |
| HVP 1251 | *GFP-cfh3::leu1⁺ ade6 his3-Δ1 h?* | This work |
| HVP 1281 | *cfh3::KAN GFP-cfh3::leu1⁺ his3-Δ1 ura4-Δ18 ade6 h⁻* | This work |
| HVP 1359 | *cfh3::KAN cdc15-GFP leu1-32 ade? his? h⁺* | This work |
| HVP 1443 | *spm1::LEU2 leu1-32 ura4-Δ1 h?* | J. Cooper |

| | | |
|---|---|---|
| HVP 1469 | *bgs4::ura4$^+$ Pbgs4::GFP-bgs4:leu1$^+$ h$^-$* | J.C. Ribas |
| HVP 1471 | *bgs1::ura4$^+$ Pbgs1::GFP-bgs1:leu1$^+$ his3-Δ1 h$^-$* | J.C. Ribas |
| HVP 1517 | *cfh3::KAN bgs1::ura4$^+$ Pbgs::GFP-bgs1:leu1$^+$ his3-Δ1 h$^-$* | This work |
| HVP 1562 | *hht2-GFP::ura4$^+$ GFP-cfh3:leu1$^+$ h?* | This work |
| HVP 1563 | *HA-cfh3::leu1$^+$ h$^-$* | This work |
| HVP 1600 | *cps1-191 cfh3::KAN leu1-32 ade6 his3-Δ1 ura4-Δ18 h$^+$* | This work |
| HVP 1706 | *cps1-191 cdc15-GFP::ura4$^+$ leu1-32 h?* | This work |
| HVP 1708 | *cfh3::KAN cps1-191 Cdc15-GFP::ura4$^+$ leu1-32 h?* | This work |
| HVP 1715 | *cfh3::KAN bgs4::ura4$^+$ Pbgs4::GFP-bgs4:leu1$^+$ h$^-$* | This work |
| HVP 1721 | *crn1-GFP::KAN ade6 leu1-32 ura4-Δ18 h$^+$* | J.C. Ribas |
| HVP 1733 | *pmk1$^+$-HA6His:ura4$^+$ ade6 leu1-32 h$^+$* | M. Gacto |
| HVP 1745 | *cfh3::KAN spm1$^+$-HA6His:ura4$^+$ ade6 leu1-32 h?* | This work |
| HVP 1747 | *cfh3::KAN spm1::LEU2 ura4-Δ18 h?* | This work |
| HVP 1775 | *cdc15-GFP:ura4$^+$ HA-cfh3$^+$::leu1$^+$ ura4-Δ18 h$^-$* | This work |
| HVP 1776 | *HA-rho1$^+$ ade6 leu1-32 h?* | P. Perez |
| HVP 1777 | *HA-rho2:KAN ura4Δ18 leu1-32 h$^+$* | P. Perez |
| HVP 1778 | *HA-cdc42:KAN leu1-32 ura4-Δ18 ade6 h$^+$* | P. Perez |
| HVP 1794 | *HA-rho1$^+$ GFP-cfh3:leu1$^+$ ade6 h?* | This work |
| HVP 1795 | *HA-rho2$^+$ GFP-cfh3:leu1$^+$ ura4Δ18 h$^+$* | This work |
| HVP 1796 | *HA-cdc42:KAN GFP-cfh3:leu1$^+$ ura4-Δ18 ade6 h$^+$* | This work |
| HVP 1797 | *cwg1-1 leu1-32 ura4-Δ18 his3-Δ1 ade6 h$^-$* | J. C. Ribas |
| HVP 1819 | *bgs1::ura4$^+$ Pbgs1::GFP-bgs1$^+$:leu1$^+$ Pcfh3::HA-cfh3$^+$:KANMX6 his3Δ1 h$^-$* | This work |
| HVP 1821 | *cfr1-GFP::ura4$^+$ HA-Bgs1::leu1$^+$ ade6 h90* | This work |
| HVP 1833 | *HA-rho1 cfh3::KAN h?* | This work |
| HVP 1845 | *end4::ura4$^+$ bgs1::ura4$^+$ Pbgs1::GFP-bgs1:leu1$^+$ h90* | This work |
| HVP 1846 | *end4::ura4$^+$ bgs1::ura4$^+$ Pbgs1::GFP-bgs1:leu1$^+$ cfh3::KAN h90* | This work |
| HVP 1850 | *cfr1-GFP::ura4$^+$ HA-Bgs1::leu1$^+$ cfh3::KAN h?* | This work |
| HVP 1955 | *cfh3::KAN crn1-GFP::leu1$^+$ his3-Δ1 ura4-Δ18 ade6 h$^-$* | This work |

**Table 4: VECTORS USED IN THIS STUDY**

| VECTOR | DESCRIPTION | ORIGEN |
|---|---|---|
| | ***Schizosaccharomyces pombe*** | |
| pAL-KS | *Amp$^r$, ori, ars1$^+$, f1, LEU2* | J.Ishiguro |
| pJK148 | *Amp$^r$, ori, f1, leu1$^+$* | McLeod et al., 1987 |
| pREP3X | *Amp$^r$, ori, ars1$^+$, LEU2* | *S. Fosburg* |
| pJCR-H 3X | *Amp$^r$, ori, ars1$^+$, f1, his3+* | J.C. Ribas |
| | ***Escherichia coli*** | |
| pBluescript$^R$ KS$^+$ | *Amp$^r$ , ColE1, lacZ, f1$^+$* | Stratagene |
| pGEM$^R$-T | *Amp$^r$, ColE1, lacZ, f1$^+$* | Promega |

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# Resumen en Español

# ANTECEDENTES Y OBJETIVO DEL TRABAJO

La levadura de fisión *Schizosaccharomyces pombe* es uno de los pocos hongos que no posee quitina en su pared celular. Sin embargo, cuando se secuenció y se analizó su genoma se encontraron varios genes que codifican proteínas con similitud a quitín sintasas o a algunas proteínas reguladores de la síntesis de quitina. En nuestro grupo estamos interesadas en estudiar la función de estos genes porque, nos pareció, que esto podría ayudar a averiguar cual es su función básica, que en *Saccharomyces* estaría adaptada a la regulación de la biosíntesis de quitina.

| Actividad | Gen de *S.cerevisiae* | Gen de *S.pombe* | Región (aa') |
|---|---|---|---|
| QSI | *CHS1* | *chs1*$^+$ (45%) | 758 |
| | | *chs2*$^+$ (29%) | 650 |
| QSII | *CHS2* | *chs1*$^+$ (47%) | 569 |
| | | *chs2*$^+$ (32%) | 643 |
| QSIII | *CHS3* | *chs1*$^+$ (28%) | 223 |
| | *CHS4* | *cfh1*$^+$ (26%) | 553 |
| | | *cfh2*$^+$ (29%) | 321 |
| | | *cfh3*$^+$ (24%) | 451 |
| | | *cfh4*$^+$ (33%) | 151 |
| | *CHS5* | *cfr1*$^+$ (32%) | 585 |
| | *CHS6* | *chs6*$^+$ (23%) | 162 |

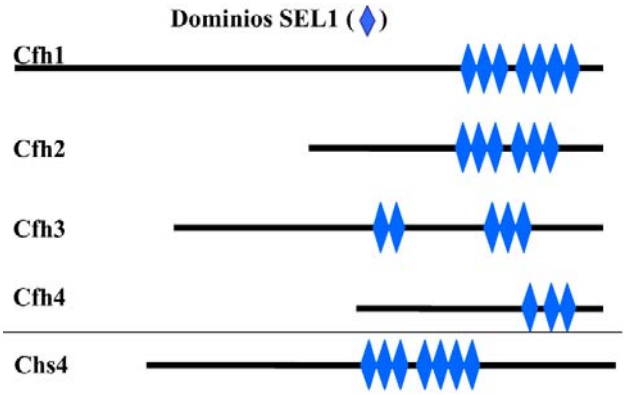
COMPARACIÓN DE LOS GENES *CHS* de *S.cerevisiae* y *S.pombe* (Porcentaje de identidad)

Así, clonamos el gen *chs1*$^+$, que era el que presentaba un mayor grado de identidad con genes de quitín sintasas y resultó que la proteína codificada por él es una auténtica enzima quitín sintasa cuya función es necesaria para la correcta maduración de las acosporas (Arellano *et al.*, 2000). El gen *chs2*$^+$ codifica una proteína con similitud a genes de quitín sintasas, pero que carece de los aminoácidos considerados el centro catalítico de estas enzimas (QRRRW) y algunos aminoácidos más que se requieren para esta función. El estudio de este gen permitió saber que la proteína Chs2p de *S. pombe* no es una enzima quitín sintasa (Martin-Garcia *et al.*, 2003) pero que juega un papel relevante en la morfogénesis de esta

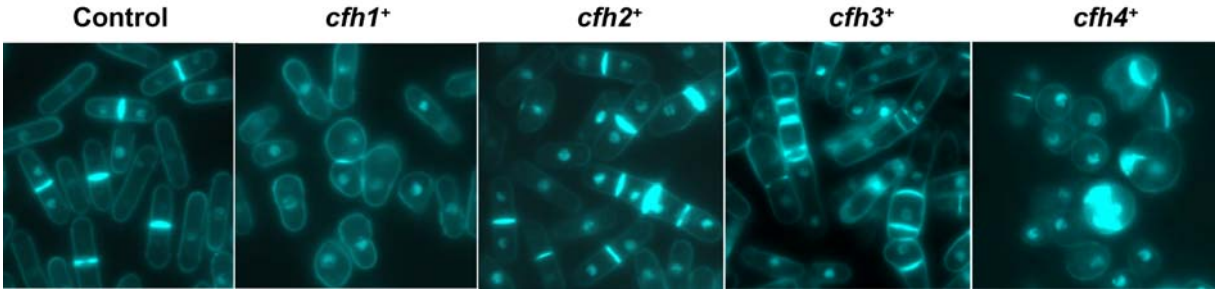
levadura al ser necesaria para mantener la estabilidad del anillo contráctil de acto-miosina (CAR, del inglés Contractile Actomyosin Ring) durante su contracción (Martin-Garcia & Valdivieso, 2006). Simultáneamente al desarrollo de este trabajo se averiguó que la proteína Chs2p de *Saccharomyces cerevisiae* era necesaria para mantener la estabilidad del CAR durante su contracción (VerPlank & Li, 2005), de modo que ambas proteínas compartían esta función, que es independiente de la síntesis de la pared celular.

A continuación estudiamos el papel de la función de Cfr1p, una proteína con similitud a Chs5p de *S. cerevisiae*. Chs5p es una proteína del Golgi tardío (TGN) necesaria para el transporte y reciclaje de la quitín sintasa Chs3p (Santos *et al.*, 1997, Santos & Snyder, 1997). Además Chs5p se requiere para la conjugación, al ser necesaria para el transporte de Fus1p (una proteína de membrana necesaria para la fusión celular durante la conjugación) a la punta de los shmoos (Santos, 2003). Cfr1p se localiza en el Golgi y no juega ningún papel aparente en la síntesis de la pared celular o la morfogénesis durante el ciclo vegetativo, pero en su ausencia hay un defecto en la fusión celular, dando lugar a un fenotipo similar al de los mutantes *chs5Δ* de *S. cerevisiae* durante la conjugación (Cartagena-Lirola *et al.*, 2006). Todos estos resultados sugerían que, en efecto, los genes de *S. pombe* similares a los genes *CHS* de *Saccharomyces* compartían con éstos una función no relacionada con la síntesis de la pared celular.

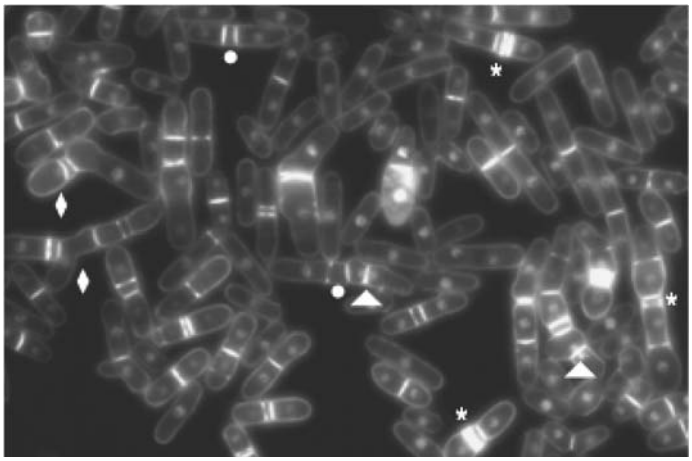
Chs4p es un regulador de Chs3p que actúa a varios niveles: es una proteína de "anclaje" necesaria para unir Chs3p a las septinas, a través de una proteína intermediaria denominada Bni4p, actúa como un activador bioquímico de la actividad quitín sintasa III y estabiliza Chs3p en la membrana plasmática (DeMarini, 1997, Trilla *et al.*, 1997, Ono N, 2000, Grabinska *et al.*, 2007, Reyes *et al.*, 2007). En el genoma de *S. pombe* hay 4 genes con similitud a *CHS4* (*cfh1*$^+$ a *cfh4*$^+$, de chs four homologue). Estas proteínas comparten con Chs4p la presencia de varias repeticiones de dominios SEL-1. Los dominios SEL-1 están presentes en proteínas que forman parte de complejos multiproteicos y que participan en procesos celulares como la ubiquitinación, el control del ciclo celular o el control de calidad de proteínas en el retículo endoplásmico (Mittl & Schneider-Brachert, 2006).




La deleción de cada uno de los genes *cfh*$^{+}$, o de los cuatro, no conduce a ningún fenotipo aparente mientras que la sobreexpresión de *cfh1*$^{+}$ o de *cfh4*$^{+}$ conduce a una pérdida de la polaridad, la sobreexpresión de *cfh2*$^{+}$ a la aparición de un pequeño número de células de morfología aberrante y la sobreexpresión de *cfh3*$^{+}$ a defectos en la citocinesis.

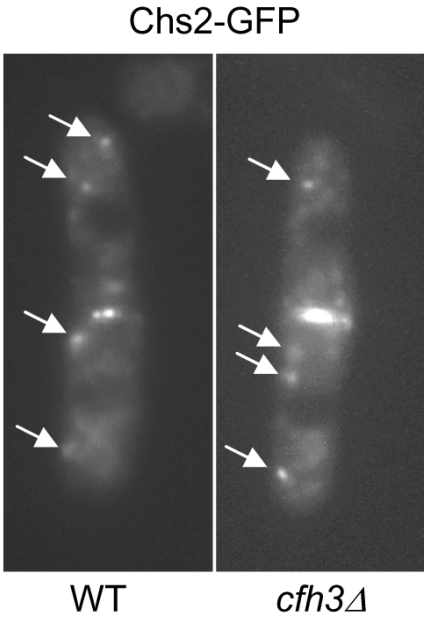



El defecto en citocinesis producido por la sobreexpresión de *cfh3*$^{+}$ recuerda al producido por la sobreexpresión de *chs2*$^{+}$, con la aparición de cadenas de células en las que se encuentran cuerpos celulares sin núcleo, ramas, núcleos atrapados por el septo y varios septos paralelos delimitando dos cuerpos celulares. Este dato sugería que Ch3p juega algún papel



en el proceso de citocinesis en esta levadura.

Matsuo y colaboradores (Matsuo *et al.*, 2004) describieron que en un mutante *cfh3Δ/ chr4Δ* Chs2p no se localiza normalmente, ya que aparece en el septo, pero también en vesículas internas. Sin embargo a lo largo de nuestro trabajo con Chs2p nosotros observamos esta proteína en vesículas internas en una estirpe silvestre con cierta frecuencia, dependiendo de las condiciones del cultivo.



En *S. pombe* no hay síntesis de quitina durante el ciclo vegetativo, no hay una proteína similar a Bni4p y las septinas no juegan ningún papel en la síntesis del septo sino en su degradación tras la citocinesis. Además dos hechos a tener en cuenta son que Chs2p es una proteína similar a quitín sintasas que participa en la citocinesis y que en *S. pombe* el septo primario está formado por β(1,3)glucano lineal, no por quitina.

A partir de estos datos surgen varias preguntas:

¿Son las proteínas Cfh reguladores de Chs2p durante la citocinesis?.

¿Están las proteínas Cfh relacionadas con las septinas y la separación celular?.

¿Regulan las proteínas Cfh la síntesis de glucano?

¿Ha tomado cada proteína Cfh cada una de estas funciones?.

¿Hay proteínas con repeticiones SEL1 formando parte de complejos multiproteicos que regulen la morfogénesis en distintos organismos?

El OBJETIVO del presente trabajo fue:
Estudiar la función de Cfh3p en la síntesis de la pared celular y en la morfogénesis de *S. pombe*.

# RESULTADOS

**Con el fin de obtener información acerca de la función que Cfh3p podría jugar en el proceso de citocinesis se llevaron a cabo las siguientes aproximaciones experimentales:**

**1. Análisis de la localización de proteínas implicadas en el proceso de citocinesis en células que sobreexpresan *cfh3*$^{+}$.** Con esta aproximación se pretendía averiguar si el fenotipo de sobreexpresión se debe a la alteración de alguna proteína en particular, lo que nos indicaría que Cfh3p podría interaccionar con esa proteína.

**2. Análisis del fenotipo de sobreexpresión en mutantes alterados en distintos pasos de la citocinesis.** La ausencia del fenotipo de sobreexpresión de *cfh3*$^{+}$ cuando cierta proteína está ausente nos indicaría que esa proteína interacciona con Cfh3p, y nos daría una idea sobre qué pasos de la citocinesis deben producirse para que Cfh3p pueda interaccionar con la maquinaria celular que controla este proceso.

**3. Localización de Cfh3p en mutantes de citocinesis.** Se pretende obtener información sobre qué proteínas son necesarias para la correcta localización de Cfh3p, lo que nos indicaría a partir de qué paso de la citocinesis ejerce su función esta proteína.

**4. Localización de proteínas implicadas en la citocinesis en un mutante c*fh3Δ*.** Esta aproximación nos indicaría si la función de Cfh3p es permitir la correcta localización de alguna proteína necesaria para la citocinesis.

**5. Análisis de las interacciones génicas entre mutantes *cfh3Δ* y mutantes en genes que participan en la citocinesis.** Se pretendía averiguar qué proteínas podrían colaborar con Cfh3p en el desarrollo de su función. Se trataba de obtener mutantes dobles con *cfh3Δ* y analizar su viabilidad, capacidad de crecimiento a distintas temperaturas y su fenotipo.

**Los resultados obtenidos a partir de todas estas aproximaciones se detallan a continuación**

### La sobreexpresión de *cfh3*$^+$ produce una alteración en la localización de proteínas implicadas en distintos pasos de la citocinesis.

Se utilizó el microscopio de fluorescencia para estudiar la localización de proteínas que participan en la citocinesis, bien mediante tinción específica (tinción de actina con rodamina-faloidina) o mediante la observación de proteínas fusionadas a la GFP. Para analizar el momento del ciclo de vida en el que se encontraban las células se realizó una tinción con Hoechst 33258, que permite la observación simultánea de los núcleos y la pared celular (ver Materials and methods) o con Calcoflúor, que permite la tinción de la pared celular. Se eligieron las siguientes proteínas para su análisis: proteínas que participan en el ensamblaje y/o la contracción del CAR (actina, Cdc4p, Rlc1p, Cdc15p y Chs2p), una proteína que es necesaria para la síntesis del septo primario (Bgs1p) y proteínas que participan en la separación celular (las septinas Spn3 y las glucanasas Agn1p y Eng1p). En cada caso se observó la localización de cada proteína en la estirpe silvestre y en células que sobreexpresaban *cfh3*$^+$. En cada caso se observaron alteraciones en la localización de la proteína analizada (ver las figuras 14-20). Este resultado sugería que Cfh3p interacciona con toda la maquinaria de la citocinesis y sugería que Cfh3p podría participar en un complejo multiproteíco.

### El fenotipo de sobreexpresión de *cfh3*$^+$ depende de la ruta SIN, de Cdc15p y de Bgs1p

Cuando las células que portan el plásmido pREP3X*cfh3*$^+$ se cultivan en ausencia de tiamina, la expresión de *cfh3*$^+$ se desreprime, de modo que se permite la sobreexpresión del mismo. Cuando esto sucede en células de la estirpe silvestre éstas presentan los problemas en citocinesis que se han descrito anteriormente. Sin embargo, cuando esto suceden en los mutantes *cdc11-119* (con una ruta SIN defectiva) o *cdc15-140* (defectivos en un componente del anillo) incubados a la temperatura restrictiva de 36ºC la células presentan el mismo fenotipo que el mutante incubado en presencia de tiamina (células alargadas sin septos; Ver la figura 21). En un mutante *cps1-191* (defectivo en la glucán sintasa Bgs1p) se observan células con un solo septo (figura 21), es decir es un fenotipo intermedio entre el del mutante *cps1-191* (sin septos) y el fenotipo de sobreexpresión de *cfh3*$^+$ (múltiples septos). Finalmente, cuando se permitió la sobreexpresión de *cfh3*$^+$ en un mutante carente de las cinco septinas se observaron células multiseptadas en las que se apreciaban múltiples septos delimitando

dos cuerpos celulares (algo característico del fenotipo de sobreexpresión de *cfh3*$^{+}$). Ver la figura 21.

En conclusión, la sobreexpresión de *cfh3*$^{+}$ requiere que la ruta SIN esté activa y que el anillo se haya empezado a ensamblar (presencia de Cdc15p), depende parcialmente de la glucán sintasa Bgs1p y no depende de las septinas (que participan en la separación celular).

### Localización de Cfh3p en mutantes de citocinesis

La proteína Cfh3p fusionada en su extremo amino terminal a la GFP se observa localizada en la zona del septo y de los polos. Una observación más detallada utilizando un microscopio confocal permitió observar que esta proteína forma un anillo que se contrae con el tiempo y que deja un rastro fluorescente en forma de placa. El patrón en anillo es típico de proteínas asociadas al CAR y el patrón en placa es típico de proteínas asociadas a la membrana plasmática. Esto último está de acuerdo con la presencia de un dominio de prenilación en el extremo carboxilo de Cfh3p.

Cuando observamos la localización de Cfh3p en mutantes afectados en distintos pasos de la citocinesis vimos que esta proteína se localiza en la zona media de las células defectivas en distintos componentes de la miosina de tipo II. En estas células Cfh3p no formaba un anillo perfectamente circular (ver la figura 22), ya que en estos mutantes el anillo no se forma adecuadamente. En las células carentes de Cdc15p no se observó Cfh3p en el ecuador celular, aunque sí en los polos celulares (figura 22). En un mutante defectivo en la ruta SIN (*cdc11-129*) Cfh3p tampoco se localiza en el ecuador celular, mientras que en un mutante que posee una ruta SIN hiperactiva la proteína permanece en la zona de los septos incluso una vez que éstos se han formado completamente (figura 22).

En resumen, la localización de Cfh3p en la zona media del anillo no depende la las miosinas de tipo II pero depende de que la proteína Cdc15p esté en dicha zona y de la actividad de la ruta SIN, lo que sugiere que Cfh3p actúa en un momento tardío del ensamblaje del anillo o durante su contracción.

### Localización de proteínas que funcionan durante la citocinesis en un mutante *cfh3Δ*

Se analizó la localización de Cdc15-GFP, Cdc4-GFP, Chs2-GFP, GFP-Bgs1p, Spn3-GFP, y Eng1-GFP en una cepa silvestre y en un mutante *cfh3Δ*. Observamos que todas estas proteínas se localizaban en la zona media de las células en ambas estirpes. Sin embargo en cierto número de células (alrededor de un 10%) las proteínas Cdc15p, Cdc4p y Chs2p parecían formar anillos que eran asimétricos o parecían estar rotos (ver más adelante).

### El mutante *cfh3Δ* muestra interacción génica con mutantes afectados en el ensamblaje del anillo contráctil y en la síntesis del septo

Se construyeron dobles mutantes entre el mutante nulo *cfh3Δ* y los siguientes mutantes: *cdc4-8*, *rlc1Δ*, *myo2-E1*, *myo3Δ*, *myo2-E1 myo3Δ* (con mutaciones en distintos componentes de las miosinas de tipo II), *cps8-188* (con una mutación puntual en el gen $act1^{+}$, que codifica la actina), *cdc11-119*, *cdc14-118* (con mutaciones en la ruta SIN), *cdc15-140*, *chs2 Δ*, y *cps1-191*. La estirpe silvestre, los mutantes sencillos y el doble mutante correspondiente a cada caso se estriaron en placas de medio rico YES y se incubaron a distintas temperaturas (22ºC, 25ºC, 28ºC, 30ºC, 32ºC, 33ºC, 35ºC, y 37ºC) durante 2-4 días. En cada caso se comparó el crecimiento del mutante doble con el de los mutantes sencillos y la cepa silvestre. El resumen de los resultados obtenidos se recoge en la tabla 2, y en la figura 23 se muestran fotografías en las que se observa cómo *cfh3Δ* muestra interacción génica con los mutantes *cdc14-118* (mutante de la ruta SIN), *cps8-188* (mutante de actina), *cdc15-140* (mutante en un componente del CAR), *imp2Δ* (mutante en una proteína necesaria para el ensamblaje y desensamblaje del CAR) y *cps1-191* (mutante en la glucán sintasa $bgs1^{+}$), ya que los dobles mutantes no crecen a temperaturas a las que los mutantes sencillos correspondientes, y el silvestre sí que lo hacen.

Del análisis de estas interacciones génicas se puede concluir que Cfh3p colabora con proteínas que actúan en el ensamblaje y/o contracción del CAR y/o en la síntesis del septo de división.

### Todos los resultados obtenidos sugerían que Cfh3p ejerce su función en el ensam-

**blaje y/o contracción del CAR o en la síntesis del septo. Con el fin de analizar más detalladamente la relación entre la proteína bajo estudio y el anillo contráctil o la síntesis del septo se llevaron a cabo varios experimentos cuyos resultados, así como las principales conclusiones obtenidas a partir de ellos, se detallan a continuación.**

***Cfh3p es una proteína asociada al anillo contráctil de actomiosina necesaria para la integridad de esta estructura, especialmente en condiciones de estrés***

En primer lugar se determinó que Cfh3p colocaliza con la actina en la zona ecuatorial de la célula, pero que una vez que Cfh3p está en esta zona, no depende de la actina para permanecer allí, ya que el tratamiento de las células con Latrunculina A (un agente que despolimeriza la actina) no hace que Cfh3p se deslocalice (figura 24). Además se determinó que Cfh3p no colocaliza con la coronina, una proteína asociada a los parches de actina (figura 24). Esto demuestra que Cfh3p no es una proteína que se asocie a todas las estructuras de actina en la célula. El hecho de que el crecimiento en presencia de Latrunculina A sea similar en el silvestre y en el mutante *cfh3Δ* confirmaron que Cfh3p no regula a la actina.

También se observó colocalización de Cfh3p con Cdc15p y además se detectó coinmunoprecipitación entre estas dos proteínas (figura 25), lo que demostró que Cfh3p es una proteína asociada al CAR. Con el fin de estudiar si Cfh3p se requería para que el CAR se ensamblara y/o se contrajera adecuadamente se llevaron a cabo experimentos de seguimiento a lo largo del tiempo ("time-lapse") utilizando cepas en las que tanto Cdc15p como la histona Hht2 estaban fusionadas a la GFP, de modo que se podía comparar las imágenes tomadas a lo largo del tiempo en los mismos momentos del ciclo de vida de las células. Inicialmente se obtuvo un tiempo de ensamblaje/contracción del anillo de 40 minutos para la estirpe silvestre y de 75 minutos para la estirpe mutante. Sin embargo, el hecho de que el mutante *cfh3Δ* no mostrara ningún defecto obvio en la citocinesis, ni en la división celular, indicaban que este resultado, aunque reproducible, era artefactual. Se pensó que esto podría deberse al modo en el que se preparaban las muestras, que incluía un paso de centrifugación y mezclar las células con medio sólido previamente fundido y mantenido a 42ºC. Cuando se prepararon las muestras recogiendo las células por filtración y extendiéndolas sobre una superficie de medio solidificado sobre el portaobjetos se obtuvo un tiempo de ensamblaje y contracción del CAR de 40 minutos para las estirpes silvestre y mutante.

Este resultado indicaba que los anillos contráctiles eran sensibles al estrés en la cepa mutante *cfh3Δ*. Para confirmar esto se cuantificaron células con una distribución anormal en las estirpes silvestre y mutante. Se cuantificaron células en las que el anillo era asimétrico (50% de los casos), aparecía roto (25% de los casos), no se desensamblaba correctamente después de la septación (20% de los casos) y células con acúmulos anormalmente intensos de Cdc15p en el citoplasma (5% de los casos).Ver la figura 26. Cuando se estimó el número de las células de cada estirpe en las que Cdc15p presentaba esas alteraciones se vio que, incluso en células incubadas en condiciones estándar (medio YES a 28ºC), la cepa *cfh3Δ* presentaba un mayor defecto en los anillos que la cepa silvestre (figura 26). Cuando las células se sometieron a distintos tipos de estrés (estrés osmótico producido por la incubación en presencia de 1,2 M sorbitol o 1.0 M KCl, estrés nutricional producido al dejar las células crecer hasta el final de la fase logarítmica o estrés mecánico, producido por centrifugación) el número de ellas que presentaban anillos anormales aumentó significativamente, siendo este número especialmente alto en las células de la estirpe mutante. Este resultado demostraba que en la cepa *cfh3Δ* los anillos contráctiles son más inestables que en la estirpe silvestre.

Seguidamente nos preguntamos si la mayor sensibilidad al estrés de los anillos en las células de la estirpe *cfh3Δ* tenía consecuencias en el crecimiento de este mutante. Para averiguarlo se inocularon diluciones seriadas de las cepas silvestre y mutante en placas de medio YES suplementado con KCl, NaCl, $MgCl_2$, cafeína o $H_2O_2$ a distintas concentraciones. Como control de crecimiento en condiciones de estrés se incluyó una cepa *spm1Δ*. Como se observa en la figura 32, los mutantes *cfh3Δ* y *spm1Δ* son más sensibles que el silvestre a KCl 1,2 M y a Cafeína 6 mM y más resistentes que el silvestre a NaCl 0,1 M. El mutante *cfh3Δ* es más sensible que el silvestre y que el mutante *spm1Δ* a $MgCl_2$ 0,1 M y crece igual que el silvestre en presencia de peróxido de hidrógeno, lo que sugiere una alteración del crecimiento en respuesta al daño en la pared celular y en respuesta a estrés osmótico, pero no en respuesta a un estrés oxidativo.

### Cfh3p regula la síntesis de glucano a través de la glucán sintasa Bgs1p.

Puesto que el mutante *cfh3Δ* presentaba interacción génica con el mutante *cps1-191*, alterado en una enzima necesaria para la síntesis del β-glucano, nos preguntamos si dicho mutante estaría afectado en la síntesis de este polímero. Esta cuestión se abordó mediante

varias aproximaciones y se encontró que el mutante *cfh3Δ* es más sensible a la Caspofungina (un inhibidor de la síntesis del glucano) que el silvestre, comportándose de modo similar al mutante *cps1-191*, y que el doble mutante *cfh3Δ cps1-191* es más sensible que los mutantes sencillos (figura 27). También se encontró que el mutante *cfh3Δ* es más sensible a la digestión por glucanasas que la estirpe silvestre, y que el doble mutante *cfh3Δ cps1-191* es más sensible que el los mutantes sencillos (figura 27). También se encontró que en los mutantes sencillos hay una ligera reducción en la cantidad de glucano presente en sus paredes celulares, respecto a la que hay en el silvestre, y que esta cantidad es significativamente inferior en el doble mutante. Estos resultados demostraron que el mutante *cfh3Δ* tiene un defecto en la síntesis del glucano. Este defecto parecía ejercerse a nivel de la subunidad catalítica de la enzima glucán sintasa, ya que la cantidad de Rho1 activo en el silvestre y el mutante son similares (figura 28).

En *S. pombe* hay cuatro genes con similitud a β-glucán sintasas (*bgs1*$^{+}$ a *bgs4*$^{+}$). Los siguientes resultados sugieren que Cfh3p regula a Bgs1p: i) una dosis alta de Cfh3p complementa parcialmente la mutación *cps1-191* (figuras 29); ii) la morfología de las células *cfh3Δ cps1-191* es similar a las de células carentes de Bgs1p mantenidas vivas con un estabilizador osmótico (figura 29) incluso a 25ºC; iii) una concentración baja de Calcofúor no tiñe los septos del mutante *cfh3Δ*, indicando que en éstos la cantidad de β(1,3)glucano lineal (para cuya síntesis es esencial Bgs1p) está significativamente reducida (figura 29); iv) Cfh3p colocaliza y coinmunoprecipita con Bgs1p (figura 30).

### En el mutante *cps1-191* los anillos contráctiles son anormales y el crecimiento en condiciones de estrés está reducido

Los experimentos explicados hasta aquí nos habían permitido saber que el mutante nulo *cfh3Δ* presenta un defecto en la integridad de los anillos contráctiles, sobre todo en condiciones de estrés, y un defecto en la síntesis de glucano. Nos preguntamos entonce si ambos fenotipos estaban relacionados o eran independientes. Para responder a esta cuestión investigamos si en el mutante *cps1-191* los anillos contráctiles y/o el crecimiento en condiciones de estrés estarían alterados.

Cuando incubamos un mutante *cps1-191* a 25ºC en medio rico YES observamos que aproximadamente el 37% de las células presentaban anillos anormales, mientras que en el

silvestre este número era del 5% y en el mutante *cfh3Δ* de un 15%. En el doble mutante este número era ligeramente superior al 40%. En todas las cepas el número de células con anillos anormales aumentaba al aumentar la temperatura del cultivo, y en todos los mutantes aumentó dramáticamente al someter las células a estrés durante 15 minutos (figura 31). Cuando observamos las células al cabo de un tiempo más largo en condiciones de estrés (2 horas) los CAR eran normales en prácticamente todas las células de todas las cepas (resultados no mostrados), es decir que las células son capaces de reparar los daños producidos en el CAR por el tratamiento con agentes inductores de estrés.

**El crecimiento defectuoso de las cepas *cfh3Δ*, *cps1-191* y *cfh3Δ cps1-191* se correlaciona con un defecto en la síntesis del β glucano.**

Seguidamente quisimos averiguar cómo era el crecimiento de las cepas *cps1-191* y *cfh3Δ cps1-191* en condiciones de estrés, en comparación con el de la cepa silvestre, el del mutante *cfh3Δ*, un mutante *cwg1-1* (que porta una mutación puntual en el gen que codifica la β-glucan sintasa *bgs4*$^+$), y el de un mutante s*pm1Δ* (carente de una MAP quinasa que participa en la ruta de integridad celular). Las placas se incubaron a 25ºC. Encontramos que los mutantes *cps1-191* y *spm1Δ* eran hipersensibles a cafeína 7 mM, que los mutantes *cfh3Δ* y *cps1-191* eran hipersensibles a $MgCl_2$ 0,2 M, que los mutantes *cfh3Δ, cps1-191* y *spm1Δ* eran hipersensibles a KCl 1,4 M (figura 32 y resultados no demostrados), y que el mutante doble era aún más sensible que los mutantes sencillos. El mutante *cwg1-1* se comportó como la cepa silvestre, sugiriendo que un defecto en *bgs1*$^+$ pero no en *bgs4*$^+$ hace que las células sean sensibles a estrés.

Se sabe que los mutantes en la ruta de Spm1/Pmk1, que controla la morfogénesis y la respuesta a estrés, muestran un defecto fuerte en citocinesis cuando crecen en condiciones de estrés. Observamos la morfología de las células de la cepa silvestre, y de los mutantes *cfh3Δ*, *cps1-191* y *cfh3Δ cps1-191* que habían estado creciendo medio sólido o en medio del líquido suplementados o no con sorbitol 1,2 M o con KCl 1.0 M a 25ºC o a 32ºC durante distintos tiempos. En cada cepa la morfología de las células crecidas bajo condiciones de estrés era similar a la morfología observada cuando las células fueron incubadas en medio YES. La morfología de un mutante doble *cfh3Δ spm1Δ* era similar a la del mutante sencillo *spm1Δ* en todas las condiciones (no mostrado). Estos resultados demostraron que la sensibilidad de los mutantes *cfh3Δ* y *cps1-191* al estrés no se debía a un defecto en citocinesis, y están de acuer-

do con el hecho de que las células eran capaces de reparar el daño en el anillo contráctil producido por el choque osmótico inicial y de adaptarse al nuevo ambiente.

Puesto que los mutantes bajo estudio mostraban un defecto en la síntesis de la pared celular, nos preguntamos si este defecto era el responsable de su sensibilidad a estrés. Para abordar esta cuestión, analizamos la composición de la pared celular de las cepas de interés en células que habían sido incubadas por 6 horas en presencia de KCl 0,6 M. Como se ve en la figura 35, todas las cepas mostraron una incorporación reducida de [$^{14}$C]glucosa en la pared celular, con respecto a los datos obtenidos cuando las células de la estirpe silvestre habían estado creciendo en medio YES (la línea discontinua en la figura representa el valor de la incorporación para la cepa silvestre en condiciones normales de crecimiento; Comparar las figuras 28 y 35). En relación con el contenido en β-glucano, la línea de puntos en la figura 35 representa su nivel en las células de la cepa silvestre incubadas en medio YES. Cuando las células fueron incubadas en YES con KCl 0,6 M, el nivel de este polímero estaba reducido un 10% en el silvestre; un 23% en los mutantes *cfh3Δ* y *cps1-191*, y un 50% en el mutante doble.

Para determinar si este efecto sobre la síntesis de la pared celular era específico para el KCl, o si era una respuesta general al crecimiento en condiciones de estrés o en un medio de una osmolaridad más alta que la estándar, analizamos la sensibilidad de las células a la digestión con glucanasas. Incubamos con 50 μg/ml de Novozyme o con 5 μg/ml de Zymolyase-100T células de las cepas silvestre, *cfh3Δ*, *cps1-191*, y *cfh3Δ cps1-191* que habían sido incubadas durante 6 horas en medio YES o en medio YES con KCl 0,6 M, sorbitol 1,2 M o $MgCl_2$ 0,125 M. En todos los casos las células se lisaron más rápidamente cuando habían sido incubadas bajo condiciones de estrés que cuando habían sido incubadas en medio YES (figura 35 y resultados no mostrados y resultados no mostrados). Estos resultados demuestran que la síntesis de la pared celular en general, y la síntesis del β-glucano en particular, se reduce perceptiblemente cuando las células están experimentando estrés, y que el crecimiento defectuoso de las cepas *cfh3Δ*, *cps1-191*, y *cfh3Δ cps1-191* en presencia de estrés se correlaciona con el bajo contenido en β-glucano de sus paredes celulares.

### *Cfh3p regula la localización de Bgs1 en la membrana plasmática*

Todos los resultados anteriores sugerían que Cfh3p es un regulador de Bgs1p y que

esta regulación es más necesaria cuando las células experimentan cierto estrés. Para entender la naturaleza de esta regulación realizamos varios experimentos. Primero realizamos Western blot para determinar el nivel de Bgs1p en las células del WT y del mutante *cfh3Δ* que habían sido incubadas en medio YES o en YES suplementado con KCl 1M durante 15 minutos. La proteína de Golgi Cfr1p se utilizó como control de carga. Según se ve en la figura 37, no había diferencia en la cantidad o la movilidad de Bgs1p en el WT o las células del mutante independientemente de si habían sufrido el choque osmótico o no. Este resultado demostró que Cfh3p actuaba en un nivel del post-traduccional.

Después, quisimos saber si Cfh3p formaba un complejo con la enzima glucán sintasa. Para aclarar esto construimos una cepa que llevaba las proteínas de fusión GFP-Bgs1 y ToRFP-Cfh3 y la observamos con el microscopio de fluorescencia. Encontramos que ambas proteínas colocalizaban (figura 30), sugiriendo que podrían estar en contacto. Para confirmar esto realizamos un experimento de la co-inmunoprecipitación usando cepas que llevaban GFP-Bgs1, HA-Cfh3 o ambas proteínas de fusión. Detectamos HA-Cfh3 en los immunoprecipitados anti-GFP en la cepa que llevaba ambas proteínas marcadas, pero no en las cepas que llevaban una sola proteína marcada. Bgs1p y Cfh3p también coimmunoprecipitaron cuando las células habían sido incubadas en presencia de KCl 1M durante 15 minutos. También analizamos la coinmunoprecipitación entre Cfh3p y Rho1p, una GTPasa que regula la actividad β-glucán sintasa. En este caso utilizamos cepas que llevaban GFP-Cfh3p, HA-Rho1p o ambas proteínas. Según se ve en la figura 30 detectamos HA-Rho1p en los precipitados de la cepa que llevaba GFP-Cfh3p y HA-Rho1p, pero no en los de las células que llevaban solamente una de las proteínas marcadas. Cfh3p y Rho1p también coimmunoprecipitaron cuando las células habían sido incubadas en KCl 1 M durante 15 minutos. Como control analizamos la coinmunoprecipitación entre GFP-Cfh3p y HA-Cdc42p (una GTPasa de la familia Rho asociada a la membrana) usando las mismas condiciones y no detectamos ninguna asociación entre estas proteínas. Todos estos resultados eran una fuerte evidencia de que Cfh3p se asocia con el complejo de la β-glucán sintasa Bgs1p. Además como habíamos visto que el nivel de Rho1 activo era similar en el silvestre y en el mutante *cfh3Δ* (figura 28) lo más probable era que esta asociación fuera relevante para la regulación de la subunidad catalítica del complejo β(1,3)glucán sintasa.

Entonces quisimos averiguar si Cfh3p regulaba la localización de Bgs1p. Para comprobar esto analizamos la localización GFP-Bgs1 en el silvestre o en el mutante *cfh3Δ* crecido

en YES o en YES con KCl 1M durante minutos. La localización de Bgs1p fue similar en ambas cepas cultivadas en YES (figura 37), aunque la señal fluorescente en las células *cfh3Δ* parecía extinguirse más rápidamente. Cuando las células fueron incubadas en presencia de KCl, la señal de GFP-Bgs1p se localizó en los polos celulares y en el septo en la cepa WT, aunque en algunas células la zona del septo aparecía deforme y la señal parecía extenderse a lo largo del polo nuevo de la célula (figura 37). Por el contrario, después del choque osmótico en el cerca de 80% de las células *cfh3Δ*, Bgs1p no se observaba en el septo ni en los polos (figura 37). Un resultado similar se obtuvo al exponer las células a sorbitol 1,2 M durante 15 minutos (no mostrado).

Para investigar si el distinto comportamiento de la proteína GFP-Bgs1 en el silvestre y el mutante era una consecuencia de un distinto comportamiento de la actina en respuesta al choque osmótico observamos la distribución de la coronina (una proteína que se asocia a los parches de actina. Crn1-GFP en la figura 37) en las cepas WT y *cfh3Δ* que habían sido expuestas a KCl 1 M durante 15 minutos. Encontramos que en ambas cepas la distribución de los parches de actina era similar, observándose en los polos y en la zona ecuatorial de las células. En ambos casos era posible detectar un cierto defecto en la morfología del área septal (flecha en la figura 37), de manera similar a lo que se observó en la cepa silvestre portadora de la proteína de fusión GFP-Bgs1 (figura 37).

Quisimos saber si la ausencia de la señal GFP-Bgs1 en las células *cfh3Δ* incubadas con KCl era debida a un defecto en la secreción de la proteína a la membrana plasmática o a su rápida endocitosis. Según se observa en la figura 37, la señal fluorescente de GFP-Bgs1 pudo observarse en los polos celulares y en la región media de la células *cfh3Δ* cuando éstas se incubaron durante 15 minutos en presencia de KCl 1 M y Latrunculin A (una droga que despolimeriza la actina, y por tanto inhibe la endocitosis) simultáneamente. Este resultado sugiere que un bloqueo en la endocytosis podría compensar la ausencia de Cfh3p. Este resultado se confirmó analizando la localización de Bgs1p en células que portaban una deleción en los genes *cfh3*$^{+}$ y *end4*$^{+}$ y que fueron expuestas a KCl 1 M durante 15 minutos (figura 37).

Para saber si Cfh3p regulaba la estabilidad de otras proteínas en la membrana plasmática observamos la localización de Bgs4p y de Chs2p en las cepas WT y *cfh3Δ* incubadas en presencia de KCl 1 M durante 15 minutos. Observamos que, en las mismas condiciones utilizadas para GFP-Bgs1p, Bgs4p se deslocalizaba de la membrana plasmática en ambas cepas,

mientras que Chs2p estaba localizado normalmente en las mismas (figura 38). Este resultado apoya la idea de que Cfh3p podría ser un regulador específico de Bgs1p. Por otro lado observamos que la localización de Cfh3p no se alteraba tras un choque osmótico (figura 39).

### *Relación entre Cfh3p y la ruta de integridad celular mediada por Spm1/Pmk1p*

El patrón de crecimiento de los mutantes *cfh3Δ* y *spm1Δ* fueron similares en algunos medios suplementados con sustancias que inducen estrés, lo que sugería que Cfh3p podría ser parte de la ruta de MAP quinasa que controla la integridad celular y la citocinesis en respuesta a estrés. Sin embargo los siguientes resultados descartaban esta posibilidad: i) los mutantes *cfh3Δ* no presentaban ningún defecto aparente en el proceso de citocinesis cuando se sometieron a condiciones de estrés (figura 34); ii) los mutantes *cfh3Δ* no mostraron el fenotipo de *vic* (de *v*iabilidad en presencia del *i*nmunosupresor y de *c*loruro; ver la figura 34). Este fenotipo es característico de mutantes en esta ruta de MAP quinasas y consiste en la capacidad para crecer en presencia de 0,2 M $MgCl_2$ y de 0,5 µg/ml de FK506; iii) en ausencia de *cfh3*$^+$ Spm1p se activa eficientemente cuando las células sufren un choque osmótico (figura 34). Esto se valoró mediante la comparación del nivel de Spm1 fosforilado (utilizando un anticuerpo anti fosfo-p42/44) respecto al nivel total de Spm1 en las células (usando un anticuerpo anti HA, ya que en la cepa utilizada la proteína Spm1 estaba etiquetada con dicho epítopo).

### *CHS4* no complementa el fenotipo del mutante *cfh3Δ*

*CHS4* es un gen de *S. cerevisiae* que posee una identidad del 24% con *cfh3*$^+$, así que nos preguntamos si este gene podría complementar el fenotipo del mutante *cfh3Δ*. Para responder a esta pregunta las cepas WT y *cfh3Δ* se transformaron con el vector pREP3X o con el plásmido pREP3X+CHS4. Para determinar la viabilidad de las células bajo condiciones de estrés, se prepararon dos lotes de placas de medio mínimo sin leucina, uno de los cuales se suplementó con $MgCl_2$ 0,2 M. Además, una placa de cada juego se suplementó con tiamina (lo que reprime la expresión de *CHS4*) y a la otra placa no se le añadió esta vitamina (condiciones de desrepresión). En cada caso, 3 x $10^4$ células que venían de cultivos en fase logarítmica de crecimiento, y tres diluciones seriadas 1:4, se inocularon en las placas correspondientes, que fueron incubadas a 32ºC durante dos días. Según se observa en la figura 41, todas las cepas podían crecer en ausencia de $MgCl_2$, independientemente de la presencia de

tiamina. Sin embargo, las células del mutante *cfh3Δ* que llevaban el vector vacío o el plásmido de sobreexpresión de *CHS4* mostraron sensibilidad a $MgCl_2$ 0,2 M, incluso en condiciones de desrepresión, indicando que *CHS4* no podía complementar el fenotipo de los mutantes *cfh3Δ*.

Con el propósito de saber si Chs4p no era funcional en *S. pombe*, o si esta proteína no podía complementar el fenotipo de los mutantes *cfh3Δ* porque no se expresaba o no se localizaba correctamente en la levadura de fisión, el mutante *chf3Δ* se transformó con el plásmido pREP3X+CHS4-GFP, en el cual la proteína Chs4p estaba fusionada a la GFP. Según se observa en la figura 41 Chs4p no se localiza correctamente de modo que la señal fluorescente se acumulada en el citoplasma y en lo que probablemente son las vacuolas, lo que demuestra que Chs4p no es estable en S. *pombe*. Este resultado indica que, aunque ambas proteínas posean una estructura similar, con varias repeticiones en tándem de dominios SEL-1, los requerimientos específicos para la localización de cada una deben ser distintos.

## Los dominios SEL-1, pero no la señal de prenilación, son necesarios para la funcionalidad de Cfh3p

Cfh3p es un miembro de una familia de proteínas que esté presente a través de la escala biológica y que se caracteriza por la presencia de varias copias de en tándem de dominios SEL-1, que son una subfamilia de los dominios Tetra Tricopéptido Repeats (TPR). Además, como muchos miembros de esta familia, Cfh3p tiene una secuencia en su extremo carboxilo (CIIS) que es un motivo potencial de prenylation. (Véase la figura 42 donde se muestra un esquema de la estructura de Cfh3p). Observamos al microscopio de fluorescencia células que llevaban la proteína Cfh3 completa, proteínas Cfh3 en las que algunas o todas las repeticiones SEL-1 se habían eliminado, o una proteína Cfh3 en la cual la secuencia del prenylation había sido eliminada, todas ellas fusionadas a la GFP. Encontramos que la eliminación de los dominios SEL-1 no tenía ningún efecto evidente en la localización de Cfh3p (figura 42 y resultados no mostrados). La proteína de Cfh3-CIIS, que carece del motivo de prenilación, se localizó en los polos celulares y en la zona ecuatorial, aunque menos específicamente que la proteína control (figura 42).

Comprobamos la importancia de estos dominios para la función de Cfh3p analizando la capacidad de las células que llevaban diversas formas de Cfh3p para crecer en placas de

medio mínimo sin leucina suplementadas con $MgCl_2$ 0,2 M e incubadas a 32ºC. Según se muestra en la figura 42 la eliminación de alguno o de todos los dominios SEL-1 abrogó la función de Cfh3p, mientras que la proteína que carecía del motivo CIIS era capaz de complementar el defecto de crecimiento en presencia del $MgCl_2$ de la cepa *cfh3Δ* (figura 42).

# DISCUSIÓN

En este trabajo se ha caracterizado la función de la proteína Cfh3p. Se ha sugerido previamente (Matsuo *et al.*, 2004) que Cfh3p/Chr4p sería un regulador de la proteína Chs2p, caracterizada por presentar similitud con quitín sintasas, pero que carece de tal actividad (Martin-Garcia *et al.*, 2003, Martin-Garcia & Valdivieso, 2006). Esto se sugirió porque aquellos autores observaron que en un mutante *cfh3Δ* Chs2p se localizaba en la zona del ecuador celular y también en vesículas internas, mientras que en una cepa control no observaban esta última localización. Sin embargo los siguientes resultados discrepan con esa conclusión: i) A lo largo de este trabajo y de trabajos anteriores nosotros hemos observado que Chs2p localiza en vesículas internas tanto en el WT como en el mutante *cfh3Δ*, dependiendo de las condiciones del cultivo (figura 43); ii) no hemos encontrado ninguna interacción génica entre *cfh3Δ* y *chs2Δ*; iii) la localización de Chs2p depende del componente de la miosinas del anillo contráctil (Martin-Garcia & Valdivieso, 2006), pero la de Chs3p no (figura 22); iv) el mutante *chs2Δ* interacciona genéticamente con mutantes de miosinas de tipo II (Martin-Garcia & Valdivieso, 2006) pero el mutante *cfh3Δ* no (este trabajo); v) la localización de Chs2p bajo condiciones de estrés es igual en el WT y el mutante *cfh3Δ* (figura 38) ; VI) las células del mutante *chs2Δ* no son sensibles a condiciones de estrés a las que las células de *cfh3Δ* sí lo son (figura 32).

Nuestros resultados apuntan a un papel de Cfh3p como proteína requerida para la estabilidad del anillo contráctil de actomiosina (CAR) y para la síntesis del β-glucano, cuya ausencia es más perjudicial para el crecimiento bajo ciertas condiciones de estrés. Hemos encontrado que en un mutante *cfh3Δ* un número significativo de células tienen los anillos contráctiles anormales y que en este mutante hay una pequeña reducción en la cantidad de β-glucano. Esta reducción es similar a la encontrada en el mutante *cps1-191*, defectivo en la enzima β-glucán sintasa Bgs1p. Aunque no se puede descartar cierta regulación de otras β-glucán sintasas, los hechos que un mutante en *bgs4*$^{+}$ no sea sensible a estrés, que una concentración baja de Calcofluor no pueda teñir los septos en el mutante *cfh3Δ*, y que la morfología de las células del mutante doble *cfh3Δ cps1-191* sea similar a la de las células de un mutante *bgs1Δ* mantenido vivo con un estabilizador osmótico (figura 29 y Cortes *et al.*, 2007) sugieren que el defecto en la síntesis del β-glucano de las células *cfh3Δ* es debido a un defecto en la actividad de Bgs1p. Es de esperar que un defecto en esta glucán sintasa resulte

en una disminución pequeña en el contenido de β-glucano, puesto que Bgs1p es responsable de la síntesis del β-glucano lineal (Cortes *et al.*, 2007), que es un componente minoritario de la pared celular (Humbel *et al.*, 2001).

*Las cepas cfh3Δ*, *cps1-191* y *cfh3Δ cps1-191* tienen una viabilidad reducida cuando el medio de cultivo está suplementado con algunos compuestos que producen estrés. Sin embargo parece ser que estas células no mueren por problemas en la citocinesis, como se ha descrito para los mutantes *spm1Δ* (Zaitsevskaya-Carter & Cooper, 1997), sino debido a una síntesis reducida de la pared celular. Una reducción en síntesis total de la pared celular también se ha observado en diversas cepas incubadas en presencia de sorbitol (Cortes *et al.*, 2005, Cortes *et al.*, 2007). Una actividad débil de Bgs1 produciría un defecto adicional, con respecto al producido por el estrés en la cepa silvestre. Así, en las células del mutante doble *cfh3Δ cps1-191* incubadas en condiciones de estrés la cantidad de β-glucano es el 50% de la cantidad de este polímero presente en las células del WT incubadas en condiciones estándar. Este defecto tan fuerte sería deletéreo para las células.

Cfh3p es una proteína asociada al anillo de Cdc15p en el CAR y al complejo de la β-glucán sintasa en condiciones estándar y de estrés. En condiciones normales de laboratorio la mayor parte de Bgs1p parece localizarse en la membrana de plasmática en ausencia de $cfh3^{+}$. En concordancia con esta observación, las células del mutante *cfh3Δ* crecen normalmente y sólo presentan una pequeña reducción en la síntesis del β- glucano lineal y un pequeño aumento en el número de anillos defectuosos cuando han sido incubadas en medio YES. Cuando las células se exponen a ciertas condiciones de estrés, ocurre un cambio en el estado físico y en las interacciones moleculares en la bicapa lipídica de las membranas, lo que influye en el ensamblaje y la dinámica de los lípidos (Kinnunen, 2000). En estas condiciones es de esperar que las proteínas transmembranales asuman nuevas conformaciones y asociaciones y que cambien sus interacciones con los lípidos de la membrana (Poolman *et al.*, 2004). Bajo estas circunstancias la β-glucán sintasa Bgs1p no se observa en la superficie celular cuando Cfh3p está ausente. El efecto del estrés se suprime cuando la endocitosis se bloquea. Así, parece que Cfh3p podría actuar como una proteína adaptadora o "andamio" para la estabilización de Bgs1p en la membrana plasmática, de modo que la célula se asegura de que la síntesis del β-glucano ocurre incluso si el ambiente no es favorable. Las células tienen un requisito más alto de β(1,3)glucano lineal en el septo. La fuerte interacción de Cfh3p con el CAR garantizaría que el septo primario se sintetice correctamente en unas condiciones desfavora-

bles, que seguramente sean frecuentes en ambientes naturales.

También observamos que en el mutante *cps1-191,* incubado en condiciones estándar de laboratorio, los anillos contráctiles son defectuosos, y que en presencia de estrés el número de células con un anillo contráctil anormal aumenta dramáticamente en las cepas *cfh3Δ*, *cps1-191* y *cfh3Δ cps1-191*. Puesto que estas condiciones parecen reducir la actividad de Bgs1p (y quizás de otras glucán sintasas), la alta frecuencia de células con anillos aberrantes en condiciones estrés es posiblemente una consecuencia del mayor defecto de Bgs1 en esas condiciones.

Sería interesante determinar si los anillos defectuosos son una consecuencia de la síntesis de septos primarios anormales o si la proteína Bgs1 por sí misma desempeña un papel directo en la estabilidad de los anillos. El hecho que el número de anillos defectuosos aumente después de un choque osmótico corto (15 minutos) señala a la última hipótesis. Aunque parezca que el defecto en la estabilidad/la estructura del anillo observado en el mutante *cfh3Δ* es una consecuencia indirecta del defecto en Bgs1p, no podemos excluir un papel específico de Cfh3p en el mantenimiento de la morfología de los anillos porque el mutante *cfh3Δ* tiene un defecto más fuerte que la cepa silvestre, y el doble mutante *cfh3Δ cps1-191* tiene un defecto más fuerte que el del mutante sencillo *cps1-191*. Además, la sobreexpresión del gen $cfh3^{+}$ produce un defecto fuerte en la citocinesis, cosa que no se observa en las células que sobreexpresan el gen $bgs1^{+}$ (Cortes, 2006).

Las características más relevantes de la proteína Cfh3p son la presencia de un sitio potencial de prenilación y la presencia de cinco repeticiones SEL-1. Estas características están también presentes en Chs4p, una proteína que regula la síntesis de quitina mediada por la quitín sintasa Chs3p en *S. cerevisiae* (Trilla *et al.*, 1997). La regulación de Chs3p por Chs4p es compleja. Chs4p es necesaria para la localización correcta de Chs3p en el cuello de la yema, anclándola a las septinas, pero también actúa como un activador bioquímico y se requiere para la estabilidad de Chs3p en la membrana plasmática (DeMarini, 1997, Trilla *et al.*, 1997, Ono N, 2000, Grabinska *et al.*, 2007, Reyes *et al.*, 2007). En *S. pombe* no hay síntesis de la quitina durante el crecimiento vegetativo (Kreger, 1954, Horisberger & Rouvet-Vauthey, 1985, Sietsma & Wessels, 1990, Arellano *et al.*, 2000), siendo el glucano el componente principal de la pared celular, y las septinas están implicadas en la separación de las células pero no en la síntesis del septo (Humbel *et al.*, 2001, Martin-Cuadrado *et al.*, 2005).

En este organismo hay cuatro proteínas con semejanza significativa a Chs4p (Matsuo *et al.*, 2004) y nuestros resultados inéditos). Nuestros resultados demuestran que una de estas proteínas (Cfh3p) desempeña un papel en la regulación de la síntesis del glucano, de acuerdo con la idea que en *S. pombe* las proteínas similares a las proteínas Chs de *S. cerevisiae* han mantenido una función general no relacionada con la síntesis de la quitina, como ya se ha demostrado para Chs2p y Cfr1p (Martin-Garcia *et al.*, 2003, Cartagena-Lirola *et al.*, 2006, Martin-Garcia & Valdivieso, 2006). En este caso, la función sería asegurar la actividad de enzimas implicadas en síntesis de la pared celular. Los requisitos específicos deben ser diferentes para Chs4p y Chs3p puesto que Chs4p no puede localizarse correctamente en la levadura de la fisión.

Hemos encontrado que el motivo de prenilación no es esencial para la función de Cfh3p. Este resultado es similar a los obtenidos para la proteína Chs4p de *S. cerevisiae* (DeMarini, 1997, Grabinska *et al.*, 2007, Reyes *et al.*, 2007). Por el contrario, la deleción de los dominios SEL-1 en Cfh3p condujo a una pérdida de la función. En *S. cerevisiae* la región de Chs4p que contiene los dominios SEL-1 puede complementar un mutante *chs4Δ* (DeMarini, 1997, Ono N, 2000, Reyes *et al.*, 2007). La región de la proteína Chs4p de *C. albicans* que contiene los dominios SEL-1 puede complementar el mutante *chs4Δ* de *S. cerevisiae* (Sudoh, 1999). Estos resultados demuestran que estos dominios son relevantes para la función de esta familia de proteínas. Se sabe que hay proteínas con este tipo de dominios que forman parte de complejos multiproteicos implicados en diversos procesos celulares, tales como el control del ciclo celular, la ubiquitinación o la degradación de proteínas en el retículo endoplásmico (Mittl & Schneider-Brachert, 2006). Nuestros resultados, junto con los encontrados en *S. cerevisiae* y *C. albicans,* demuestran que hay complejos de proteínas en los que participan proteínas con dominios SEL-1 que juegan un papel relevante en la síntesis de la pared celular y la morfogénesis en diversos organismos.

# CONCLUSIONES

1. La proteína Cfh3p regula a la enzima β(1,3)glucan sintasa Bgs1p a nivel post-traduccional.

2. Cfh3p y Bgs1p son necesarias para garantizar la estabilidad del anillo contráctil de actomiosina.

3. La función de Cfh3p es más necesaria cuando las células están sometidas a condiciones de estrés.

4. En *Schizosaccharomyces pombe* la síntesis de glucano disminuye cuando las células están sometidas a condiciones de estrés.

Mirza Mohammad Reza SHARIFMOGHADAM

توانا بود هر که دانا بود

ز دانش دل پیر برنا بود