9 Chitin Synthesis and Fungal Cell Morphogenesis

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CONTENTS

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I. Introduction

Chitin is an extremely rigid natural polysaccharide and, hence, one of the most important structural biopolymers on earth. It constitutes an essential part of the fungal cell wall, where it is required for the maintenance of cell integrity. Interestingly, its importance does not only derive from its structural role as the major component of the primary septum (PS) but also from its function as a scaffold for the assembly of other components of fungal walls. The interconnections between chitin and other cell wall components determine cell morphogenesis, which is necessarily influenced by environmental changes. The variety observed in cell morphologies and environmental niches across the fungal kingdom would explain the high diversity of chitin synthases (CSs) present there, as well as their different regulatory mechanisms, making chitin synthesis a paradigm for studies addressing fungal morphogenesis. In addition, studies aimed at characterising the regulation of chitin synthesis are the basis for the development of new antifungal therapies.

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II. Chitin Synthases and Deposition of Chitin at the Fungal Cell Wall

After cellulose, chitin is the second most abundant biopolymer. This polymer is present in the fungal cell wall, the exoskeleton of arthropods and some other animal structures. Chitin synthesis is carried out by **chitin synthases (CSs)**, a family of enzymes that use uridine-diphospho-*N*-acetylglucosamine (UDP-GlcNAc) as a substrate and catalyse the $2n$ UDP-GlcNAc→[GlcNAc-β(1,4)-GlcNAc]$_n$+$2n$ UDP reaction. Oligosaccharides are not reaction intermediates. In fungi, chitin synthesis takes place at the plasma membrane (Durán et al. 1975) in a vectorial fashion (Cabib et al. 1983), taking the substrate from the cytoplasm while the nascent fibrils are extruded to the outside of the cell, where the polysaccharide is located. Chapter 8 also covers fungal cell wall polysaccharides. CSs are assumed to be organised in supramolecular complexes at the plasma membrane (PM) in order to facilitate the assembly of chitin fibres. The length of the chitin chains synthesised in vivo and in vitro ranges between 120 and 170 units in *Saccharomyces cerevisiae* (Orlean 1987), the organism in which chitin synthesis has been best characterised. Several CS activities with different in vitro requirements have been described; thus, *Sc*CSI has an optimum pH of 6.5 and is stimulated by the presence of GlcNAc and of Mg^{2+}; Co^{2+} and Ni^{2+} cations inhibit this activity. *Sc*CSII activity levels are approximately 5 % of those of CSI; this enzyme has an optimum pH of 7.5–8.0. Co^{2+} stimulates activity better than Mg^{2+}, while Ni^{2+} inhibits the reaction. Finally, *Sc*CSIII activity is approximately 10 % of the CSI activity level; Mg^{2+} is the best stimulating cation, and neither Co^{2+} nor Ni^{2+} inhibits it. These biochemical differences between the three *S. cerevisiae* CS activities are exploited to determine each of them in the presence of the other two [see Cid et al. (1995) and Roncero (2002) for revision]. CSs from *Candida albicans* also differ in their optimal pH and are stimulated by divalent cations. *Ca*CSII is more active in the presence of Mg^{2+}, while *Ca*CSI and *Ca*CSIII seem to respond slightly better to Co^{2+} (Kim et al. 2002). Recent reports show that the presence of *N*-acetylchito-tetra, -penta and -octaoses in the reaction increases the initial velocity of *Sc*CSI activity, although the nature of this activation is not well understood (Becker et al. 2011). Gyore et al. (2014) have shown that chemically modified analogues of GlcNAc can act as primers in *Sc*CSII in vitro reactions. However, the requirement for a natural primer in the reaction has not been conclusively shown. CSs from other fungi have been found to have similar biochemical characteristics, with requirements for divalent cations and free GlcNAc for optimal activity.

In *S. cerevisiae*, chitin accounts for only 2–3 % of the cell wall dry weight, but it is not uniformly distributed along the cell wall. Most of the chitin accumulates at the septum that will form the bud scar after cytokinesis, whereas the remainder is scattered throughout the cell wall (Molano et al. 1980). Septum synthesis is a complex process that starts early in the cell cycle [see Orlean (2012) for a recent revision]. As soon as a new bud emerges, a chitin ring is synthesised at the base of this bud. As the daughter cell grows, the ring remains at the neck between mother and daughter cells. After mitosis, when nuclei have segregated, more chitin is formed, starting at the ring and continuing centripetally while the plasma membrane invaginates. When the plasma membranes merge, the primary septum (PS) is completed, forming a disc-like structure within a thickened chitin ring. Chitin synthesis at the primary septum is performed by the activity of CSII, while chitin synthesis at the ring and lateral wall is carried out by CSIII. Later, the secondary septum, whose composition is similar to that of the rest of the cell wall, is laid down at both sides of the PS (see Fig. 9.1a for a schematic drawing of the yeast septum). At cytokinesis, the two cells separate through the action of a chitinase (see below) in an asymmetric way; most of the chitin remains in the mother cell, giving rise to the bud scar. A less conspicuous structure—the birth scar—remains on the surface of the daughter cell. Then, the newborn bud starts a process of maturation, which includes growth and some cell wall remodelling. From this moment onwards,

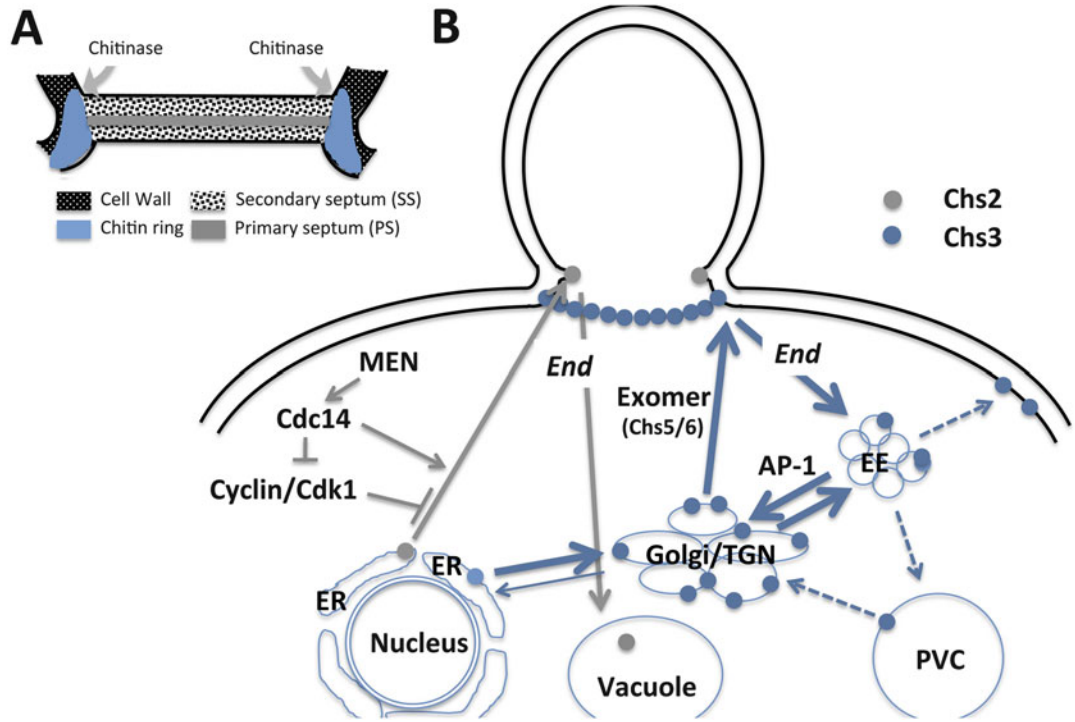

Fig. 9.1 Chitin synthases direct the assembly of yeast septa. (**a**) Structure of the yeast septum showing the two major chitin structures, the chitin ring and the PS. Cell separation occurs after cytokinesis by the action of chitinases from the daughter cell side. (**b**) Traffic and cellular localisation of major yeast CSs, Chs2p and Chs3p. Chs2p travels in a cell cycle controlled form from the ER to the PM at the neck, where it synthesises the PS; later, it is endocytosed and degraded. Chs3p exits the ER and is sorted at the Golgi/TGN boundary towards the PM by the exomer. Chs3p becomes anchored through Chs4p/Bni4p (not shown) to the septin ring to synthesise the chitin ring, being later endocytically recycled back to the Golgi/TGN by means of the AP-1 complex. The alternative routing of Chs3p to the plasma membrane in the absence of AP-1 is indicated by *dashed arrows*. *MEN* mitotic exit network, *ER* endoplasmic reticulum, *TGN* trans-Golgi network, *End* endocytosis, *EE* early endosomes, *PVC* prevacuolar compartment

chitin appears interspersed throughout the lateral wall of the growing bud (Shaw et al. 1991).

In filamentous fungi, chitin is also deposited at the septa, although most of this polymer is distributed uniformly along mycelia after chitin deposition at the tip of the growing hyphae.

III. Chitin Synthases

A. The Diversity of Fungal Chitin Synthases: A Common Catalytic Centre for Multiple Functions

The diversity of fungal CSs was initially uncovered by PCR analysis using degenerated oligonucleotides based on *S. cerevisiae* and *Candida albicans CHS* sequences (Bowen et al. 1992). Since then, many other fungal CS sequences have been identified in more than 200 fungi, and they have been analysed using different comparison algorithms. The number of CS genes identified per species ranges between two and nine, and a detailed study of the multiple CS genes has revealed that three sequence motifs—QXXEY, EDRXL and QXXRW—and seven isolated residues in the core region are conserved in all CS genes [revised in Ruiz-Herrera et al. (2002)]. The QXXRW domain is also present in other glycosyltransferases (GT) (Saxena et al. 1995), and it has been proposed to be the catalytic domain for GT2 enzymes, including CSs. Accordingly, mutations in the

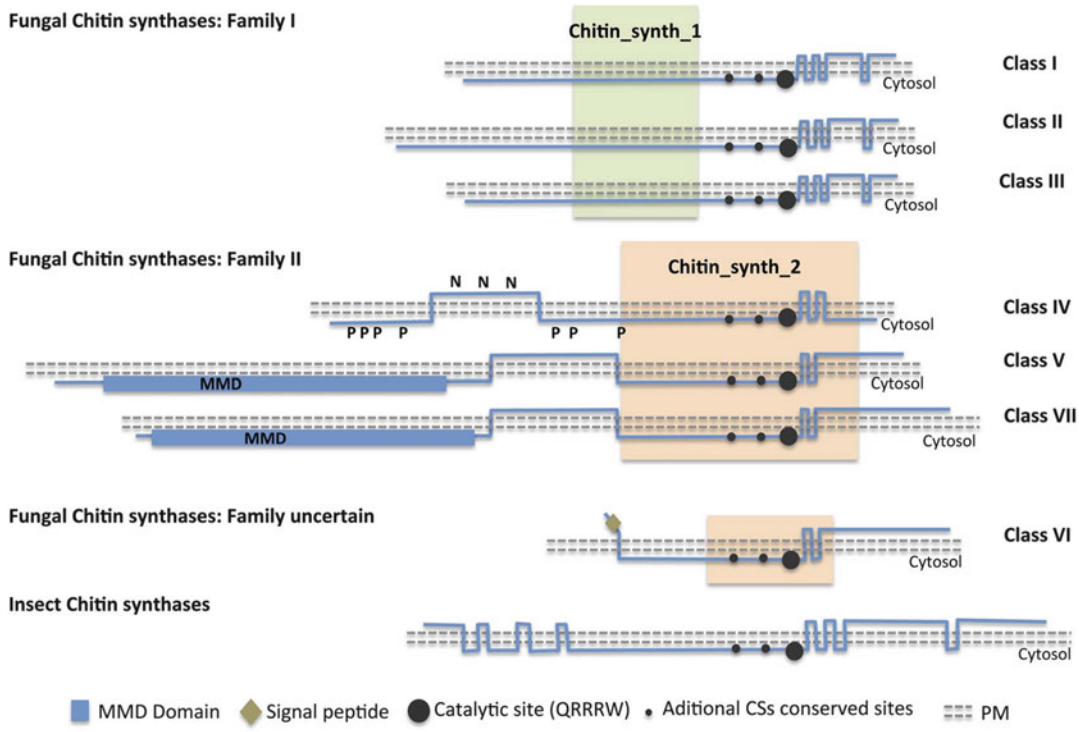

Fig. 9.2 Schematic representation of the different CS classes based on their predicted secondary structure obtained from *S. cerevisiae* and *A. fumigatus* proteins. The images are drawn at approximate scale. *Dashed grey lines* represent the plasma membrane (PM). CS sequences are represented by a *blue line* in which vertical sections represent transmembrane regions. *Shaded boxes* mark the Chitin synthase 1 and 2 domains as described in Pfam. Representative domains are indicated in the figure; note the positional conservation of putative catalytic sites (*large filled circles*). Secondary structure predictions have been only confirmed for *Sc*Chs3p; the N-glycosylation and phosphorylation sites experimentally verified for this protein are included in the scheme

bona fide chitin synthase QRRRW domain result in a loss of both function in vivo and enzymatic activity in vitro in all *S. cerevisiae* CSs (Nagahashi et al. 1995; Cos et al. 1998). Moreover, the *chs2*$^{+}$ gene from *Schizosaccharomyces pombe*, which lacks these conserved domains, does not code for any CS activity (Martin-Garcia et al. 2003). The consensus sequences identified maintain the same structural features and the same relative location with respect to one another, pointing to their relevance for the proper conformation of the enzymes (Ruiz-Herrera et al. 2002).

The conclusions drawn from multiple analyses have led to the classification of CSs in two large families/divisions, subdivided into seven classes (Roncero 2002; Lenardon et al. 2010). **Family I** genes share the so-called chitin_synth_1 motif (Pfam database accession number: PF01644), and this family includes the *ScCHS1/2*, *CaCHS1/2* and *CaCHS8* genes. All its members show the catalytic domain flanked by a C-terminal region containing multiple transmembrane (TM) domains (Fig. 9.2). The inclusion of additional fungal sequences in multiple comparison analyses allowed the classical subdivision of this family into classes I, II and III. A phylogenetic analysis within the family strongly supported the later appearance of class III enzymes (Ruiz-Herrera and Ortiz-Castellanos 2010); however, a precise separation between Family I enzymes is extremely difficult owing to the high similarity between them and to the lack of functional conservation between the three classes across fungi (see below).

Family II includes the homologues of the *ScCHS3* and *CaCHS3* genes. The genes in this family share the chitin_synth_2 motif (PF03142) and a significant degree of similarity, including a catalytic domain always flanked on both sides by TM regions (Fig. 9.2). Thus, their secondary structure is clearly distinct from the proteins in Family I, a distinction that is also supported by functional assays. While *S. cerevisiae* and *C. albicans* only contain a single member of this family, most fungi contain at least three. Members of this family have been separated into different classes, depending on the author; as a consequence, the subdivision of Family II into classes could be confusing. Class IV, which is present in all fungal groups, is recognised as a unique class in this family, and it usually contains a single member. However, basidiomycetes contain two highly similar members of this class, probably originated by a gene duplication event in the group. Class V CSs are typical of filamentous fungi and are notably absent from the *Saccharomycetales* (yeast) group [see Wang et al. (2009) for a revision on fungal phylogeny]. Based on their catalytic domains, class IV and V CSs are highly related enzymes, although class V CSs contain a myosin motor-like domain (MMD) in their N-terminal region that is absent in class IV members. Further studies separated class V enzymes into two different CS classes that differ in their MMD domains and in their physiological relevance, thus leading to the current separation into class V and VII enzymes. In most cases, the genes encoding these proteins are linked to the chromosome in a head-to-head configuration, sharing their promoter regions; this suggests a common evolutionary origin. The MMD domain is required for the actin-based localisation of these enzymes to sites of active chitin synthesis during hyphal growth. The distinct absence of class V/VII CSs in the *Saccharomycetales* group is probably due to a gene loss effect, in all probability associated with the fact that they only grow as yeasts.

Additional CSs genes have been described in filamentous fungi. Based on sequence comparisons and secondary structures, these CSs seem to define the new class VI of CSs with an uncertain position between Families I and II. However, the experimental evidence about this class is limited, and hence the suggestion of a new family, as recently proposed, should await further studies (Gandia et al. 2014).

B. The Ancient Evolutionary Origin of Chitin Synthases

Although chitin synthesis is best characterised in fungi, CS genes have been also found in Diatomea, Choanoflagellates and several Metazoa (Ruiz-Herrera and Ortiz-Castellanos 2010; Merzendorfer 2011; Zakrzewski et al. 2014). In a recent systematic analysis of *CHS* genes, the authors found that enzymes from Choanoflagellates and Metazoa can be grouped into two clades [I and II; Zakrzewski et al. (2014)]. Clade I is more homogeneous and includes enzymes from all the groups analysed; chitin synthases grouped in the ancient class A from insects belong to this clade. Clade II is a paraphyletic group with a more complex evolution that includes the *CHS2* genes from the insect class B and from nematodes. Interestingly, *CHS* genes from Metazoa exhibit a core region with the chitin_synth_2 motif (Fig. 9.2), suggesting a closer relationship with Family II of CSs (Roncero 2002; Merzendorfer 2011; Zakrzewski et al. 2014). Finally, CS-like genes have also been identified in bacteria, where chitin oligomers participate in nodulation (Peters 1997). The *Nod*C gene from *Sinorhizobium meliloti* also shows significant similarity with Family II, suggesting a very ancient origin for the genes that cluster in this family. Based on these data, it is possible to propose an evolutionary trend in chitin synthases. Their origin would be very old, starting with a gene already present in bacteria and probably related to the current Family II of *CHS* genes. Ancient gene duplication at the base of the fungal kingdom would have originated the two fungal *CHS* families reported to date, which now have somewhat different biological functions. The only gene of Family I would have also duplicated very early, and hence all fungi contain class I and II genes. More recent duplication in filamentous fungi would have led to the third class currently observed in Family I. In specific cases, such as

in *Aspergillus fumigatus*, more recent duplications would have produced isoenzymes within the same class. A similar situation can be found in Family II, in which duplication from an initial ancestor present in all fungi (class IV) would have led to the class V genes in filamentous fungi after the acquisition, by genetic recombination, of the MMD region; a later duplication would have originated classes V and VII. Interestingly, some metazoan chitin synthases also contain a MMD domain, apparently originated in a fully independent evolutionary event (Zakrzewski et al. 2014). This suggests that the incorporation of MMD domains to CSs would be a common evolutionary trend in the regulation of chitin synthases. An interesting example of evolutionary trends in fungal CSs can be found in *S. pombe*, a relatively modern yeast in which there are two *CHS*-like genes but only one of them (*chs1*$^{+}$) is functional (Arellano et al. 2000; Martin-Garcia et al. 2003). *chs1*$^{+}$ belongs to class I and only synthesises minute amounts of chitin during sporulation. Since vegetative cells do not contain appreciable amounts of chitin, it is not surprising that other *CHS* genes have been lost along the evolution of this fission yeast.

It should be noted, however, that the current classification of CSs is mostly based on sequence comparison and therefore does not take into account the different secondary structure shown by Families I and II of fungal CSs and by metazoan CSs. Moreover, the function of most of the CSs is not yet fully understood (see below); accordingly, the complete evolutionary story of chitin synthases is still unwritten.

IV. Chitin Synthesis and Fungal Cell Wall Assembly

A. Chitin Synthesis and Cell Wall Assembly

Chitin localises to the inner part of the cell wall, close to the PM [see Lesage and Bussey (2006), Orlean (2012), and Cabib and Arroyo (2013) for reviews]. Considerable efforts have been made to characterise the connections between the different polymers of the yeast cell wall. Initially, the existence of a β(1,4)-linkage between the reducing GlcNAC terminus of a chitin chain and the nonreducing terminal glucose of β(1,3)-glucan was described (Kollar et al. 1997). Later, it was reported that all the cell wall components were associated, forming a complex in which β(1,6)-glucan occupied a central position; chitin and β(1,3)-glucan were directly attached by glycosidic linkages, whereas mannoproteins were joined to the polysaccharide through the lipidless remains of a glycosylphosphatidylinositol anchor. Despite this, a complex was described that lacked the mannoproteins (Kollar et al. 1997), and Pir proteins (Proteins with internal repeats) were found to be linked to β(1,3)-glucan through an alkali-sensitive linkage (Ecker et al. 2006). More recently, it has been found that one fraction of chitin is linked to β(1,3)-glucan, another to β(1,6)-glucan, while a third one remains free. These linkages depend on the localisation of chitin (Cabib and Duran 2005); chitin in the PS is mostly free, with only a small fraction linked to β(1,3)-glucan. Chitin dispersed throughout the lateral walls is mostly attached to β(1,6)-glucan. Finally, chitin in the ring of early budding cells is mostly attached to β(1,3)-glucan chains. The presence of alkali-insoluble high-molecular weight β(1,3)-glucan attached to the chitin ring controls morphogenesis at the mother-bud neck by preventing local cell wall remodelling (Blanco et al. 2012). This static fraction of β(1,3)-glucan would keep the width of the neck constant during cell division, allowing the proper segregation of organelles and cell division (Cabib and Arroyo 2013). All these results suggest the existence of different modules that join to form a lattice whose characteristics vary with location and would be influenced by internal and external signals. Several studies have identified the transglycosidase activities involved in the formation of cell wall interlinkages. ScCrh1p and ScCrh2p are Glycosylphosphatidylinositol (GPI)-anchored proteins, with homology to the glycosidase hydrolase family 16. They localise to the cell wall sites where chitin is present, acting as in vivo transglycosylases that form the linkages

between chitin and both β(1–3)- and β(1–6)-glucans (Cabib and Arroyo 2013). *CHR1*/2-dependent activities are also required for maintaining the Gas1p transglycosylase at the neck, where it contributes to cell wall assembly by forming additional inter-glucan linkages (Rolli et al. 2009).

Structural studies in *C. albicans* cell wall composition indicate that there are no significant differences between the hyphal and yeast forms, except for a fourfold increase in chitin content in hyphae [reviewed in Klis et al. (2001) and Chaffin (2008)]. The cell wall structure of *C. albicans* contains similar polymers and linkages to those described for *S. cerevisiae*, hinting that the overall cell wall organisation is similar in both organisms. However, there is evidence that in *C. albicans*, β(1,6)-glucan can bind chitin through the C6 position of the GlcNAc residues, a linkage that has not been found in *S. cerevisiae*. Antiparallel chitin chains can bind through hydrogen bonds, resulting in highly insoluble structures. These linkages produce a meshwork of structural fibrillar polysaccharides to which GPI-anchored and Pir cell wall proteins bind covalently. Additionally, there are proteins embedded in the cell wall that are non-covalently bound to the polysaccharides [see Klis et al. (2001) and Chaffin (2008) for reviews].

All these assemblies lead to the formation of the fungal cell wall in which septum synthesis is considered to be a paradigm of fungal morphogenesis (Cid et al. 1995; Cabib et al. 2001; Cabib and Arroyo 2013). In yeast, the chitin ring contributes to septum assembly, while the synthesis of PS is the critical point in the cytokinesis process. Although there are significant differences among organisms, in yeast (and probably in all fungi) the synthesis of the chitin forming the PS is concomitant with the contraction of an actomyosin ring [see Roncero and Sanchez (2010) and Bi and Park (2012) for reviews], both processes being highly interdependent. Mechanistically, the process seems to have been well conserved across fungi, even in organisms lacking chitin such as *S. pombe*, where the role of chitin in PS synthesis has been replaced by the linear β(1–3)-glucan made by the *bgs1*$^{+}$ glucan synthase (Cortes et al. 2007).

Much less is known about cell wall assembly in other forms of fungal growth. Hyphae exhibit a tubular morphology because growth takes place at their tips, where the cell wall is constantly being remodelled. Chitin is usually more abundant in hyphae than in yeasts and, together with other polymers, is continuously incorporated to the cell wall while new bonds are being formed. Studies by Wessels et al. (1983) suggest that the newly-formed chitin is not fully organised, which renders it more susceptible to enzymatic remodelling. As the hypha grows, chitin is crosslinked to glucans at the subapical zone, increasing cell wall rigidity. In *A. fumigatus* chitin, galactomannan and the linear β(1,3) β(1,4)-glucans are covalently linked to the nonreducing end of β(1,3)-glucan side chains while β(1,6)-glucan is absent in this microorganism, as well as in most fungi (Free 2013). Chitin is linked via a β(1,4)-linkage to β(1,3)-glucan [see Latge et al. (2005) for a review on the *A. fumigatus* cell wall]. Chapter 8 also covers *Aspergillus* cell wall polysaccharides. Recent data suggest that chitin synthesis is critical as a scaffold for cell wall assembly during hyphal extension [see below and Jimenez-Ortigosa et al. (2012)]. Septum synthesis during mycelial growth is a poorly known process, although data on *C. albicans* indicate that septum closure in hyphae also depends on chitin, suggesting a mechanism similar to that reported for yeast. The potential cell wall changes during fungal conidiogenesis have not yet been extensively studied, but chitin synthesis seems to be an essential part of this process since many *chs* mutants (see below) show significant defects in sporulation. Moreover, chitin has been shown to be an important component of conidial cell walls. In *S. cerevisiae* spores, chitin is deacetylated into chitosan (Christodoulidou et al. 1999), which serves as a scaffold for the dityrosine outermost layer that confers the characteristic strength of spores. Similarly, chitin is an essential component of *A. fumigatus* conidial cell walls, where it also performs scaffold functions

necessary for its characteristic rodlet coverage (Jimenez-Ortigosa et al. 2012).

B. Chitin Degradation and Cell Wall Assembly

Chitinases hydrolyse the β(1,4)-glycosidic bonds of chitin, contributing actively to fungal cell wall remodelling. In *S. cerevisiae*, the *Cts1p* chitinase is required for cell separation while *Sc*Cts2p participates in sporulation [see Adams (2004) for a review on fungal chitinases]. The daughter cell directs cell separation through the asymmetric expression of some genes (including *CTS1*) from the Ace2p transcription factor [reviewed in Lesage and Bussey (2006)]. The potential excess in chitinase activity is normally buffered through cell wall assembly mechanisms; however, deregulation of *CTS1* becomes critical after some cytokinesis defects have emerged, including the absence of the otherwise marginal CSI activity (Cabib et al. 1992; Gomez et al. 2009). A similar function has been described for the *CHT3* chitinase in *C. albicans* (Dunkler et al. 2005). Moreover, inhibition of this activity seems to occur during hyphal growth to prevent mycelium fragmentation (Gonzalez-Novo et al. 2008). An additional family of chitinases similar to *ScCTS2* has been tentatively implicated in sporulation in different organisms (Dunkler et al. 2008). Filamentous fungi have several potential chitinases in their genomes, although no morphogenetic role has been assigned to any of them and most of them probably have nutritional functions (Alcazar-Fuoli et al. 2011).

C. Chitin Synthesis in Response to Cell Stress

As an essential component of the fungal cell wall, chitin synthesis is regulated by stress. In yeasts, cell wall damage triggers a compensatory mechanism to guarantee cell survival; the most apparent effect of this mechanism is the synthesis of chitin-rich amorphous salvage septa after failures in PS synthesis (Shaw et al. 1991). Moreover, cell wall damage caused either by external agents, such as caspofungin, zymolyase or calcofluor, or by mutations in cell wall-related genes triggers a compensatory response mediated by the Cell Wall Integrity (CWI) signalling pathway [reviewed in Levin (2011)]. According to the data accumulated in *S. cerevisiae* and *C. albicans*, this response is known to be complex and interconnected with other signalling routes. Full coverage of this response is out of the scope of this manuscript, but it is worthwhile noting that an essential part of this response is the increase in chitin synthesis mediated by alterations in the intracellular traffic of CSs (see below) and also by the upregulation of *ScGFA1*, which code for a glutamine:fructose-6-phosphate amidotransferase involved in the synthesis of metabolic precursors for chitin. The latter effect is believed to act after cell damage by funnelling metabolic fluxes to the synthesis of the substrate for CSs, increasing chitin synthesis and cell wall strength (Lagorce et al. 2002). Accordingly, *ScGFA1* overexpression can alleviate the otherwise lethal effect of some cell wall-related mutations (Gomez et al. 2009). These compensatory mechanisms not only challenge the efficiency of some antifungal drugs directed against the cell wall (Munro 2013; Walker et al. 2013) but also offer new opportunities for exploring synergistic treatments combining chitin synthase inhibitors with other antifungal agents.

V. A Single Polymer but Distinct Functions for Chitin Synthase Enzymes

A. The Biological Function of Family I CSs

In eukaryotic cells, a contractile ring of actin, type II myosin, and many other proteins assemble underneath the plasma membrane before the end of mitosis. This assembly defines the site at which cytokinesis will occur. Once cells have segregated their nuclei, the actomyosin ring contracts; this contraction is coupled to plasma membrane ingression (Wloka and Bi 2012). Yeast cells need to coordinate actomyosin ring contraction with the deposition of

the primary septum that forms centripetally between the dividing cells. Cells then synthesise secondary septa on both sides of the primary septum, before digesting partially the primary septum to allow cell separation (Roncero and Sanchez 2010).

1. Chitin Synthase I

It is after cell separation when the class I CS CHS1 performs its function in *S. cerevisiae* (Cabib et al. 1989). *chs1*Δ mutants are viable, exhibiting very mild phenotypes under non-stressed conditions. ScChs1p acts as a repair enzyme that synthesises a minor fraction of the cell wall chitin in the bud scar immediately after cytokinesis to overcome the possible damage caused by excessive chitinase activity. In fact, disruption of *ScCTS1*, the chitinase required for cell separation, suppresses the lysis associated with *chs1*Δ cells (Cabib et al. 1989, 1992). *C. albicans* has two class I CS enzymes: *CaCHS2* and *CaCHS8*. Disruption of *CHS8* alone or in conjunction with a disruption in *CHS2* generates mutant cells with normal morphology and growth rates in both the yeast and hyphal forms. However, both enzymes have been shown to contribute to chitin synthesis in vivo and to the synthesis of salvage septa in the absence of other CSs (Lenardon et al. 2007; Walker et al. 2013).

2. Chitin Synthase II

In *S. cerevisiae*, formation of the primary septum is carried out by CSII, whose catalytic subunit is Chs2p. At the end of the cell cycle, Chs2p synthesises the primary septum, which grows centripetally, contributing (together with actomyosin ring contraction) to plasma membrane ingression and separation of the two cells (Schmidt et al. 2002). Thus, cells have developed regulatory mechanisms to ensure that primary septum formation will be coordinated with cell cycle events and will only occur once cells have segregated their chromosomes and have assembled their actomyosin ring (Wloka and Bi 2012). Deletion of *CHS2* is either lethal or causes extremely severe defects during cytokinesis, depending on the genetic backgrounds, because the primary septum is not synthesised in *chs2Δ* mutants (Sburlati and Cabib 1986; Shaw et al. 1991). The viability of *chs2Δ* mutants depends on the chitin produced by CS III, which promotes the assembly of a highly abnormal salvage septum (Schmidt et al. 2002; Cabib and Schmidt 2003). Assembly and contraction of the actomyosin ring promote efficient septum deposition at the correct location. Conversely, septum deposition modulates the dynamics and stability of the contractile ring during cytokinesis. Therefore, the assembly and contraction of the actomyosin ring and septum deposition are interdependent processes, since defects in one of them clearly affect the other (Bi 2001; Schmidt et al. 2002; VerPlank and Li 2005). The function of *ScCHS2* orthologue in *C. albicans* (*CaCHS1*) is clearly conserved, since lack of *CaCHS1* has deleterious effects on cell viability due to alterations of essential processes such as primary septum formation (Munro et al. 2001; Lenardon et al. 2010). Similarly to *S. cerevisiae chs2Δ* mutants, *C. albicans chs1Δ* cells induce alternative forms of septation that rescue the lethal cell division defect produced by the absence of *CaCHS1* (Walker et al. 2013).

3. Other Class I, II and III Fungal CSs

While the function of Family I CS in yeast seems straightforward, as described above, the function of these enzymes in filamentous fungi is much more uncertain. Single and double mutants in class I, II and III enzymes have been constructed in several fungi. Their characterisation has led to the conclusion that Family I enzymes always account for most of the CS activity in vitro, as has been described for yeast. However, the morphogenetic phenotypes reported for these mutants vary, depending on the species, making it extremely difficult to obtain an overall view of the biological function of these enzymes. Individual class I and II mutants have marginal or null phenotypes in several fungi, but the characterisation of some double mutants points to a potentially redundant role for both classes in cell wall assembly

(Ichinomiya et al. 2005). In any case, the defects observed are fairly mild, even in the double mutants. The only notable exception seems to be *Yarrowia lipolytica*, whose class II enzymes seem to perform functions in septum assembly (Sheng et al. 2013) similar to those reported in *S. cerevisiae* and *C. albicans*. This exception is probably linked to the intermediate evolutionary position of this dimorphic fungus (see above). By contrast, deletion of class III enzymes in several filamentous ascomycetous fungi produces dramatic morphological alterations. These effects were initially described in *Neurospora crassa* (Yarden and Yanofsky 1991) and later in multiple systems, such as several *Aspergillus* species (Borgia et al. 1996; Mellado et al. 1996; Muller et al. 2002), *Penicillium* (Liu et al. 2013), *Magnaporthe* (Kong et al. 2012), or *Botrytis* (Soulie et al. 2006). However, there is no conclusive experimental evidence pinpointing the precise role of this class of CS in cell wall assembly. Surprisingly, to date no phenotype for class III enzymes has been described in basidiomycetous fungi.

In light of all these results, it seems that, with the unexplained exception of basidiomycetes, Family I CSs perform an essential function in fungal cell morphogenesis. This function has apparently been displaced from class II enzymes in yeast to class III in filamentous fungi, a process probably associated with the evolutionary diversification between Family I chitin synthases.

B. The Biological Function of Family II CSs

1. Chitin Synthase III (Class IV)

The function of class IV chitin synthases became clear after the initial characterisation of the *Scchs3Δ* mutant, which contained as little as 10 % of the chitin content of a wild-type strain. This result was a clear indication that in *S. cerevisiae* class IV, CSIII activity was the major in vivo activity in budding yeast. Most of this chitin forms a ring that surrounds the neck constriction at the mother side. This ring is not essential for cell survival but performs a homeostatic function during cytokinesis, and it becomes an essential structure when other neck assembly functions are compromised. In extreme cases, S*c*Chs3p is able to synthesise salvage septa that functionally replace the absence of PS (Cabib 2004). A very similar function for *CaChs3p* has been described in the dimorphic yeast *C. albicans* (Walker et al. 2013), where *CaChs3p* also proved to be responsible for most of chitin synthesis during hyphal growth. Class IV enzymes have also been described as the major in vivo CSs in other yeast-like organisms such as *Ustilago maydis* (Weber et al. 2006), *Y. lipolytica* (Sheng et al. 2013), *Wangiella dermatitidis* (Wang et al. 1999) or *Cryptococcus neoformans* (Banks et al. 2005), although in the latter this chitin is mostly deacetylated into chitosan, which forms part of the capsular polysaccharide. Interestingly, some of these organisms are dimorphic, exhibiting both the yeast and hyphal forms. This makes it possible to test the importance of class IV enzymes in hyphal development. Unfortunately, all the class IV mutants characterised in these systems lacked apparent phenotypes during mycelial growth and hence no relationship between class IV CSs and hyphal development has been found other than that demonstrated for *C. albicans*, where Chs3 is involved in that process {Sanz et al. 2005 #1405}. Additional characterisation of class IV mutants in true filamentous fungi, such as *N. crassa*, *A. nidulans*, *A. fumigatus* or *M. oryzae*, did not provide additional information because none of the mutants exhibited an altered phenotype, even after extensive characterisation. Surprisingly, some of these "nonfunctional" class IV enzymes have been shown to have functional catalytic sites based on heterologous expression (Jimenez et al. 2010). Accordingly, their role in chitin synthesis currently remains a mystery.

2. Chitin Synthases with Myosin Motor-Like Domain

To a certain extent it could be expected that the other Family II CS members (the class V/VII enzymes, only present in filamentous fungi) could perform a major role in chitin synthesis during mycelial growth. Extensive characterisa-

tion of multiple class V/VII mutants failed to provide conclusive evidence of a direct involvement of these enzymes in bulk chitin synthesis. However, all class V/VII mutants characterised to date show morphogenetic defects, probably associated with cell wall alterations. Moreover, most class V/VII mutants characterised are hypersensitive to cell wall-related drugs, such as calcofluor white or caspofungin, and most class V mutants are hypovirulent in their correspondent hosts [see Madrid et al. (2003), Weber et al. (2006), and Kong et al. (2012), for specific references]. In addition, it has recently been shown that AfcsmA and AfcsmB mutants have reduced in vitro chitin synthase activity (Jimenez-Ortigosa et al. 2012). The picture that emerges from these data is that class V/VII CSs would participate in chitin synthesis, although their contribution to the total amount of chitin is low. The phenotypes observed in these mutants suggest that this chitin might become a critical anchor for other cell wall polymers, such that its absence would contribute to the significant cell wall alterations reported. Most class V/VII mutants also show sporulation defects, suggesting a specific role for these enzymes in cellular differentiation. Moreover, the class V *AfCsmA* mutant has recently been shown to have significantly reduced chitin levels in its conidial cell wall, which triggers alterations in the conidial surface (Jimenez-Ortigosa et al. 2012). This observation seems to be the first conclusive evidence of a direct role of class V enzymes in chitin synthesis.

The biological relevance of the genetic duplication that led to class V and VII homologues in fungi is uncertain. CSs from both classes have been described to have redundant functions in *A. nidulans* due to the synthetic lethality of the double V/VII mutant (Takeshita et al. 2006); however, this result has not been reproduced in any other fungi studied, including the close relative *A. fumigatus* (Jimenez-Ortigosa et al. 2012). In all other fungi studied, class V mutants show the strongest phenotypes, although additional deletion of the corresponding class VII gene exacerbates the defects. Therefore, from the studies undertaken to date it may be concluded that class V and VII enzymes perform partially redundant functions, class V enzymes being more important in biological terms. In this scenario, it is tempting to speculate that a direct relationship between the Family II CSs and chitin synthesis might be masked by compensatory effects between the different activities. However, this possibility seems unlikely since no clear alteration in the transcription patterns of these genes has been found in the mutants in which this hypothesis has been analysed.

In sum, although further analyses are required, the studies undertaken to date have revealed the morphogenetic importance of Family II CS across the fungal kingdom. Class IV enzymes perform essential functions during yeast growth that are important both quantitatively and qualitatively for cell wall assembly. Their biological roles have apparently been replaced by class V/VII enzymes during hyphal growth and sexual differentiation, with a relevance that is more qualitative than quantitative. Both roles highlight the importance of chitin as a scaffold for other more abundant cell wall polymers and the critical relevance of chitin–glucan linkages in fungal cell wall assembly and fungal morphogenesis, as recently proposed (Cabib and Arroyo 2013).

C. The Elusive Function of Class VI CSs

The biological function of fungal class VI CSs remains as controversial as its evolutionary relationship with Families I and II (see above). Mutants in this class do not show any apparent morphological or physiological phenotype, although they do have reduced virulence in some fungal pathogens, such as *B. cinerea* and *M. oryzae*. In *M. oryzae*, this trait has been linked to a specific defect in appressorium formation during the initial step of infection (Kong et al. 2012). Thus, despite the scarce information, class VI enzymes could possibly be associated with specialised functions in fungal plant pathogens.

VI. Regulation of Chitin Synthases

An Overview CSs have been recognised for decades as integral membrane proteins. In view of this, a critical question in the field has been to

determine where and how chitin synthases are activated, assuming that chitin synthesis along the secretory pathway could be catastrophic. According to the results of in vivo experiments, the early view of CSs as zymogenic enzymes activated by proteolytic processing no longer holds. The work carried out in *S. cerevisiae* has led to the understanding that the regulation of chitin synthesis is essentially a question of the intracellular transport of the CS enzymes to the plasma membrane (PM) and their potential interaction with other proteins at the cell surface. Interestingly, Family I and II CSs differ considerably in their secondary structures (see Fig. 9.2), even though they are able to synthesise a similar polymer. This indicates that the most conserved region of these enzymes (the catalytic domain followed by a complex transmembrane region) should suffice for directing chitin polymerisation at the PM, while the additional regions of these proteins would be involved in their regulation and/or traffic, processes that are essentially different in both families (see below). In any case, the existence of specific activators for the different CSs at the PM cannot be fully excluded, although their hypothetical biochemical roles remain unknown.

An additional and very important question regarding the regulation of fungal chitin synthesis is the relevance of the coupling between polymerisation and crystallisation. This coupling was reported many years ago (Roncero and Durán 1985) and should be dependent on homo- or hetero-oligomerisation of CSs into supramolecular complexes, as has been demonstrated for cellulose synthases (Atanassov et al. 2009). Unfortunately, the experimental evidence for fungal CS oligomerisation is scarce and very recent (Sacristan et al. 2013). Thus, no clear conclusions can yet be inferred regarding its importance in the regulation of chitin synthesis.

A. Regulation of Family I CSs

1. Regulation of Chitin Synthase I

A cell cycle regulation analysis using synchronised *S. cerevisiae* cultures indicated that the expression of *ScCHS1* mRNA peaks at the M/G1 transition, although the *Sc*Chs1 protein is fairly stable and its level does not change significantly during the cell cycle. It has been suggested that Chs1p would be transported in specialised vesicles, the so-called chitosomes, from where it would be activated and located at the PM (Chuang and Schekman 1996; Spellman et al. 1998). In *C. albicans*, a transcription analysis during the cell cycle revealed that the expression of *CaCHS8* peaked in G2, whereas the expression of *CaCHS2* was non-periodic (Cote et al. 2009). The expression of *CaCHS2* increased shortly after the induction of hypha formation, and both enzymes seem to be required to synthesise chitin during hyphal growth (Gow et al. 1994; Munro et al. 2001, 2003).

2. Regulation of Chitin Synthase II

The activity of *Sc*Chs2p is essential for the synthesis of the primary septum that is laid down behind the contractile ring during cytokinesis (Shaw et al. 1991). The expression, localisation and enzymatic activity of *Sc*Chs2p are temporally and spatially regulated to ensure that septum formation proceeds only after all chromosomes have been pulled apart (Choi et al. 1994; Chuang and Schekman 1996; Spellman et al. 1998; VerPlank and Li 2005). CSII is deposited at the division site through the secretory pathway a few minutes before mitotic spindle breakdown. The delivery of *Sc*Chs2p occurs just before and during actomyosin ring contraction (Chuang and Schekman 1996; VerPlank and Li 2005; Fang et al. 2010). Unlike *ScCHS2* regulation, there is no deep insight into the regulation of its orthologue in *Candida*. The expression of *CaCHS1* peaks in G2, which corresponds to its function in the synthesis of the primary septum during cell division (Munro et al. 2001; Cote et al. 2009).

Cyclin-Dependent Kinases (CDKs) govern progression through the cell cycle. Cdks bind to specific regulators called cyclins, and kinase activity associated with different cyclin/Cdk complexes promotes phase-specific events. At the end of the cell cycle, mitotic forms of Cdk must be down-regulated to allow cells to exit

from mitosis, perform cytokinesis and start a new cell cycle (Sanchez-Diaz et al. 2012). The kinase Cdk1 (encoded by *ScCDC28*) is the master regulator of the cell cycle in the budding yeast *S. cerevisiae* and controls *Sc*Chs2p dynamics. Indeed, *Sc*Chs2p is a substrate of Cdk1 in vivo, and *Sc*Chs2p is phosphorylated at the N-terminus of the protein (Chin et al. 2012). During mitosis, when Cdk activity is still high, *Sc*Chs2p is synthesised and held at the endoplasmic reticulum (ER) precisely through its phosphorylation by Cdk1 at four consensus Cdk1-phosphorylation sites in its N-terminal tail (Zhang et al. 2006; Teh et al. 2009).

Once chromosomes have been segregated at the end of mitosis, budding yeast cells activate a signalling pathway, the so-called Mitotic Exit Network (MEN), which consists of a small GTPase, Tem1p and protein kinases Cdc15p, Dbf2p and Dbf20p, together with a few regulatory proteins (Meitinger et al. 2012). The main goal of MEN is the release of the conserved phosphatase Cdc14p from the nucleolus to the entire cell to dephosphorylate key substrates, which then drives cells out of mitosis through an efficient and rapid cytokinesis and the start of a new cell cycle (Bouchoux and Uhlmann 2011; Meitinger et al. 2012; Palani et al. 2012; Sanchez-Diaz et al. 2012). In order to promote exit from mitosis, the phosphatase Cdc14p induces the inactivation of mitotic Cdk activity by dephosphorylation, and hence activation, of the Cdk inhibitor Sic1p, its transcription factor Swi5p, and Cdh1p, an activator of the Anaphase Promoting Complex (APC) that induces the degradation of mitotic cyclin. In parallel, ScChs2p can be dephosphorylated by Cdc14p, which determines *Sc*Chs2 export from the ER (Chin et al. 2012). In fact, it has been shown that Cdc14p promotes the capture of ScChs2p into COPII transport vesicles at the ER (Zhang et al. 2006; Jakobsen et al. 2013). In addition, the dephosphorylation of *Sc*Chs2p stimulates interaction with the COPII component Sec24p, which is considered to be responsible for binding to membrane cargo proteins at the ER, concentrating them in the forming vesicles (Jakobsen et al. 2013). Subsequently, *Sc*Chs2p is delivered to the division site via the secretory pathway using actin cables and type V myosin, together with the exocytic machinery (Chuang and Schekman 1996; VerPlank and Li 2005).

The assembly of the cytokinetic machinery in budding yeast is a sequential and orchestrated process that starts with the assembly of a septin ring early in the cell cycle (Wloka and Bi 2012). Soon after the formation of a new bud, the type II myosin Myo1p forms a ring at the bud neck that later on during mitosis associates with other factors. Among them are actin-nucleating and bundling factors such as formins and the Iqg1p protein, which promote the assembly of the contractile actomyosin ring at the end of anaphase (Wloka and Bi 2012). Components of the actomyosin ring interact with *Sc*Chs2p and play a critical role in regulating PS formation. At the onset of cytokinesis, the septin ring is split into two cortical rings, which act as diffusion barriers at the division site during cytokinesis (Dobbelaere and Barral 2004), while *Sc*Chs2p and actomyosin ring components are sandwiched between the hourglass cortical rings. By FRAP (Fluorescence Recovery After Photobleaching) analysis, it has been shown that *Sc*Chs2 initial localisation is fairly dynamic and its delivery is completed in a few minutes after its initial arrival (Wloka et al. 2013). Soon after, *Sc*Chs2p becomes immobile during cytokinesis, and its immobility depends on Myo1p (Wloka et al. 2013).

Localisation at the cleavage furrow seems to be insufficient for *Sc*Chs2p to synthesise the PS (Nishihama et al. 2009), since *Sc*Chs2p must be activated in vivo by a poorly understood mechanism that can be mimicked in vitro by protease treatment. Indeed, the proteolysis of cell membranes containing *Sc*Chs2p by trypsin stimulates the chitin activity associated with *Sc*Chs2p in vitro (Sburlati and Cabib 1986; Martinez-Rucobo et al. 2009), suggesting that *Sc*Chs2p requires further activation once it has been deposited at the division site. This activation defines an additional level of control to ensure the appropriate coordination of actomyosin ring contraction, PM ingression and PS formation (Schmidt et al. 2002).

Recent findings suggest that the actomyosin ring components Hof1p, Inn1p and Cyk3p coordinate these events during cytokinesis

(Sanchez-Diaz et al. 2008; Jendretzki et al. 2009; Nishihama et al. 2009). *Sc*Chs2p interacts directly with Hof1p and stabilises it at the cleavage site. In addition, Hof1p binds to Myo1p, which could contribute to coupling actomyosin ring contraction to primary septum formation (Oh et al. 2013). It also appears that Cyk3p could regulate CSII activity, since an increased dosage of Cyk3p stimulates *Sc*Chs2p-dependent chitin synthesis and the formation of PS-like structures at the bud neck (Meitinger et al. 2010; Oh et al. 2012). However, Cyk3p plays a dual role during cytokinesis since it inhibits secondary septum formation while simultaneously promoting primary septum deposition (Onishi et al. 2013).

Moreover, another key factor in the regulation of *Sc*Chs2p activity is Inn1p, a protein originally described to be essential for the coordination of actomyosin ring contraction, plasma membrane ingression and primary septum deposition (Sanchez-Diaz et al. 2008; Nishihama et al. 2009). Inn1p associates with the actomyosin ring and co-purifies with Hof1p, Iqg1p and Cyk3p (Sanchez-Diaz et al. 2008; Nishihama et al. 2009; Palani et al. 2012; Nkosi et al. 2013). Regarding the dynamics of *Sc*Chs2p during cell division, the localisation and protein–protein interactions of these factors are controlled by CDK phosphorylation and subsequent dephosphorylation by Cdc14p, which highlights the key role of Cdc14p during cytokinesis (Meitinger et al. 2010; Sanchez-Diaz et al. 2012). Indeed, Cdc14p-dependent dephosphorylation of Inn1p contributes to Inn1p–Cyk3p complex formation (Palani et al. 2012). In Inn1p-depleted cells, the contracting actomyosin ring appears to be unstable and often collapses, similar to the phenotype of cells lacking *Sc*Chs2p (VerPlank and Li 2005; Sanchez-Diaz et al. 2008). Inn1 protein has a C2 domain at the amino terminus of the protein that is essential for ingression of the plasma membrane, whereas the remainder of the protein is required for the timely localisation of Inn1p at the bud neck (Sanchez-Diaz et al. 2008). It has recently been described that hypermorphic versions of *Sc*Chs2p suppress the defects in PM ingression produced by an inactive form of the C2-domain of Inn1p (Devrekanli et al. 2012). These dominant suppressor mutations in ScChs2p are located at conserved sites in the catalytic domain and exhibit enhanced CS activity, suggesting that the ScChs2p mutations suppress the loss of Inn1 function through its enhanced CS activity. Moreover, Inn1p associates with *Sc*Chs2p in yeast cell extracts. Together, these results suggest that Inn1p might directly regulate the CS activity associated with *Sc*Chs2p at the division site (Devrekanli et al. 2012).

After its role during exit from mitosis, MEN kinases regulate cytokinesis by acting on components of the contractile actomyosin ring (Meitinger et al. 2012). *Sc*Chs2 protein is unable to efficiently localise to the division site in the absence of MEN activity, and chitin deposition is therefore reduced (Meitinger et al. 2010). It has been suggested that such a defect could be mediated via Cyk3p, which is unable to localise to the bud neck in the absence of MEN function (Meitinger et al. 2010). In addition, Dbf2 kinase directly phosphorylates *Sc*Chs2p and triggers its dissociation from the actomyosin ring during the late stage of cytokinesis (Oh et al. 2012). Soon after actomyosin ring contraction has ended, *Sc*Chs2p undergoes internalisation in endosome-like vesicles and is degraded in the vacuole, depending on the major vacuolar protease Pep4p (Chuang and Schekman 1996). Direct phosphorylation of *Sc*Chs2p would facilitate its separation from the ring and subsequent endocytic removal from the division site (Oh et al. 2012).

3. Regulation of Other Class I, II and III Fungal CSs

Very little work has been carried out to decipher the in vivo CS regulation of these enzymes in other fungi, and the results reported are mostly descriptive. *Neurospora crassa* proteins *Nc*Chs1, *Nc*Chs3 and *Nc*Chs6 have been shown to be transported to the sites of new cell wall synthesis in a type of specialised vesicles called chitosomes. Microscopy evidence suggests the existence of distinct populations of vesicles, whose transport depends on actin but not on microtubules, as inferred from the use of drugs

interfering with actin or microtubule assembly (Riquelme et al. 2007; Sanchez-Leon et al. 2011). The peculiarities of this transport have led some authors to propose that CSs might be transported by a route other than the classical pathway from the ER via the Golgi apparatus to the plasma membrane (Riquelme et al. 2007).

B. Regulation of Family II CSs

1. Regulation of Chitin Synthase III

The characterisation of calcofluor resistance in *S. cerevisiae* has provided an extensive list of proteins involved in CSIII (class IV) activity. While *Sc*Chs3p is the catalytic subunit, the other proteins are markers of an intracellular transport route that delivers ScChs3p to the PM in a polarised way, allowing chitin synthesis at the neck constriction. The characterisation of this route has made *Sc*Chs3p a paradigm for the study of the intracellular traffic of proteins. While a detailed description of this transport is out of the scope of this chapter, its essential rules are described succinctly below.

The exit of *Sc*Chs3p from the ER depends on a dedicated ER membrane chaperone named Chs7p (Trilla et al. 1999; Kota and Ljungdahl 2005), also being modulated through *Sc*Chs3p oligomerisation (Sacristan et al. 2013) and palmitoylation (Lam et al. 2006). Together, these factors allow the progression of *Sc*Chs3p through the Golgi, preventing its recycling back to the ER (Sacristan et al. 2013). After ER exit, Chs3p populates the designated Golgi/Trans Golgi Network (TGN) boundary, where proteins are classified for their delivery to the PM. *Sc*Chs3p exit from Golgi/TGN depends on the Chs5p and Chs6p proteins, which form a specialised transport complex named exomer and required for polarised transport of Chs3p (see Fig. 9.1b, full lines) (Santos and Snyder 1997; Trautwein et al. 2006; Wang et al. 2006). Chs6p is one of the four Chs5p- and Arf1p-binding proteins (**ChAPs**), a set of homologous proteins that associate with the Chs5p scaffold and might assemble in alternative exomer complexes involved in the transport of different proteins to the PM (Trautwein et al. 2006; Rockenbauch et al. 2012). During the last part of its transit to the PM, Chs3p interacts with Chs4p, which leads to CSIII activation at the PM through an unknown mechanism (Reyes et al. 2007). In addition, Chs4p serves as a Chs3p anchor to the neck through its interaction with Bni4p and the septin ring (DeMarini et al. 1997; Kozubowski et al. 2003; Sanz et al. 2004). This anchor regulates the onset of Chs3p endocytosis, regulating chitin synthesis (Sacristan et al. 2012). Upon endocytosis, Chs3p is recycled to the Golgi/TGN by a complex mechanisms not yet fully understood that mostly relies on the AP-1 complex (Valdivia et al. 2002). This recycling prevents Chs3p degradation in the vacuole and allows the maintenance of a massive reservoir of Chs3p at the Golgi/TGN, from where Chs3 can be mobilised upon cellular request. Deletion of the AP-1 complex in an exomer-defective background activates an alternative route (see Fig. 9.1b, dashed arrows) for *Sc*Chs3p delivery to the PM (Valdivia et al. 2002; Starr et al. 2012). Alterations in this transport allow cells to regulate the amount of Chs3p present at the PM to increase chitin synthesis after environmental stress; this is well documented, at least after cell wall (García-Rodríguez et al. 2000) or temperature (Valdivia and Schekman 2003) stress. Not surprisingly, post-translational modification of Chs3p contributes to the regulation of its traffic and to the regulation of chitin synthesis [Lenardon et al. (2010) and our unpublished results].

The rules governing the transport of Family II CSs in other fungi have not been studied extensively, but the fact that all these CSs are localised to the growing tips and septa suggests the existence of mechanisms for polarised transport. Interestingly, the proteins involved in the transport route described above for the class IV *CHS3* enzyme are well conserved across the fungal kingdom, with the notable exception of *S. pombe*, which lacks the otherwise omnipresent and well-conserved Chs7 protein (Fig. 9.3). This absence probably reflects the proposed coordinated evolution of class IV CSs and Chs7 proteins (Jimenez et al.

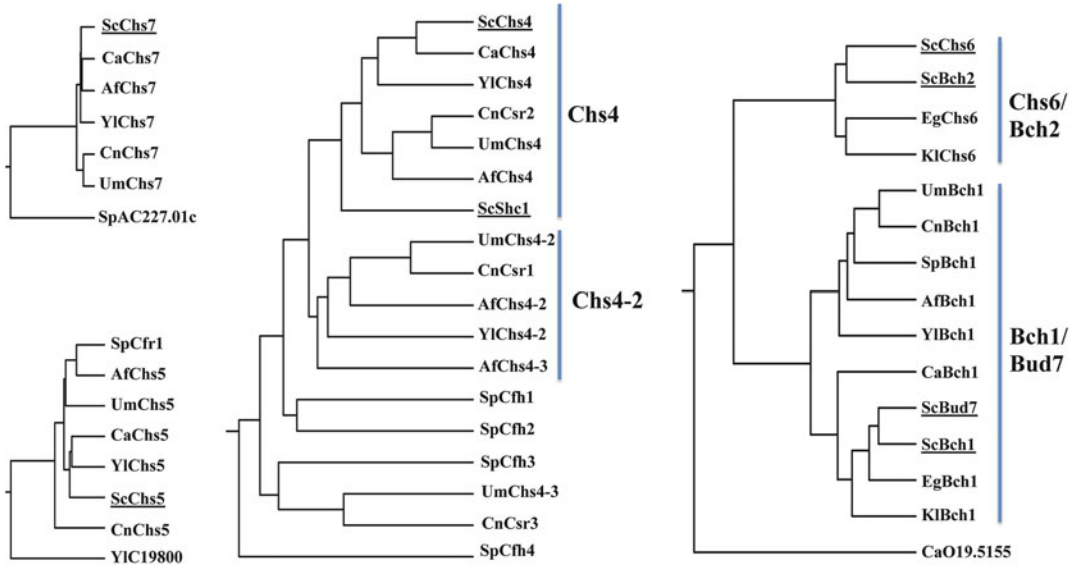

Fig. 9.3 NJ rooted phylogenetic trees after clustal W analysis of proteins homologous to *Sc*Chs4, *Sc*Chs5, *Sc*Chs6 and *Sc*Chs7. Analysis was performed with sequences from *A. fumigatus, C. neoformans*, *U. maydis*, *Y. lipolytica*, *S. cerevisiae*, *C. albicans*, *K. lactis*, *E. gosypii* and *S. pombe*. All sequences with significant similarity after BLAST searches were included in the analysis, acting the most divergent sequence as outgroup for the rooting. Note the high degree of conservation between the Chs5 and Chs7 proteins, but the divergence between Chs4 and Chs6 proteins that led to the separation in well-defined subgroups. For comparison purposes, all the trees are represented at the same scale. See text for additional explanations

2010), both of which are absent in *S. pombe*. *ScCHS7* is well conserved evolutionarily and performs similar functions in *S. cerevisiae* and *C. albicans*, allowing Chs3p export from the ER (Trilla et al. 1999; Sanz et al. 2005). Strikingly, *CHS7* does not seem to be required for the transport of the class V/VII enzymes from the ER (Martin-Urdiroz et al. 2008; Jimenez et al. 2010).

The **exomer** is a well-conserved complex in fungi, as suggested by the observation that *CHS5* and *CHS6* orthologues are present in all of them [Trautwein et al. (2006), Fig. 9.3]. Chs5p is the essential component of the exomer, its N-terminal region being sufficient and necessary for its function (Martin-Garcia et al. 2011; Paczkowski et al. 2012). Not surprisingly, this region has been extremely well conserved along evolution (Fig. 9.3). Comparison of the ChAPs (Chs5- and Arf1-binding proteins) hints at a fascinating story about exomer evolution. Most fungal ChAPs group together with the *Sc*Bch1/*Sc*Bud7 paralogues (Fig. 9.3), suggesting the very early origin of these proteins and probably also a conserved function within the exomer. However, analysis of sequences from the fungal post-WGD (whole-genome duplication) clade and related genera among the Saccharomycotina (Wang et al. 2009) supports an early duplication event between the ChAPs that led to the appearance of the *ScCHS6* homologues which, according to the work undertaken in *S. cerevisiae*, would be specialised in the transport of CSs. Later on, *ScBCH1* and *ScCHS6* would have duplicated into *ScBUD7* and *ScBCH2*, respectively, because of the well-documented whole-genome duplication that occurred in the bona fide WGD clade. This duplication would have in turn led to a further specialisation of the different ChAPs in *S. cerevisiae*, the exomer always acting as a kind of specialised complex in the polarised transport of different cargos (Trautwein et al. 2006; Barfield et al. 2009; Ritz et al. 2014). These studies raise several questions regarding the exomer, such as whether it is involved in chitin synthe-

sis in all fungi, whether it is always involved in polarised transport and how many cargos are really dependent on the exomer.

Unfortunately, the exomer has been studied in detail only in *Saccharomyces*. There is some, but not much, information from the distantly related yeast *S. pombe* that suggests that the exomer might be conserved mechanistically (Martin-Garcia et al. 2011). However, the data from this fission yeast do not link it to polarised transport [Cartagena-Lirola et al. (2006) and our unpublished results]; instead, the *S. pombe* exomer might play some minor role in intracellular traffic. Thus, the answers to these questions will require the characterisation of exomer components in other systems.

Meanwhile, the extensive work that is currently ongoing in *S. cerevisiae* will probably shed light on the reasons for the extreme ChAPs specialisation observed in this organism.

The Chs4 protein is required for chitin synthesis in *S. cerevisiae*, *C. albicans* and *C. neoformans* (Trilla et al. 1997; Ono et al. 2000; Banks et al. 2005), a clear indication of the conserved function of this protein in the regulation of class IV CSs. Moreover, *S. cerevisiae* contains a paralogue (*SHC1*) that is regulated differentially during sporulation (Sanz et al. 2002). Fungal CHS4 orthologues can be unambiguously separated into two distinct groups, one of which has been lost along evolution in the Saccharomycotina group. Unfortunately, there is no information about the physiological function of the second group, although recently the truncation of *A. nidulans* AN3445, the true orthologue of *AfCHS4-3*, has been shown to confer calcofluor resistance (He et al. 2014), raising the possibility of a broad function of these proteins in cell wall assembly. The demonstration of a direct role for them in chitin synthesis will require additional characterisation.

2. Regulation of Fungal Chitin Synthases During Mycelial Growth: Chitin Synthases with a Myosin Motor-Like Domain

As described above, the function of class IV CSs in filamentous fungi is minor or null. Therefore, the most relevant issue has been to determine how the transport of class V/VII enzymes (both having a myosin motor domain in their N-terminal region) is achieved during hyphal growth. For class V enzymes, it has been well documented that both the MMD and CS domains are required for its function; it was therefore assumed that the MMD domain serves for the polarised transport of these enzymes along the hyphal axes (Takeshita et al. 2005). However, the detailed work carried out by Steinberg's group in *U. maydis* suggested that the mechanism was more complicated than anticipated (Treitschke et al. 2010; Schuster et al. 2012). *Um*Mcs1p moves back and forward to the hyphal tip through the cytoskeleton; it seems that this movement is independent of the MMD domain, which would be specifically required for the final actin-dependent translocation of Mcs1p into the PM, a process that affects only 15 % of the Mcs1p molecules arriving at the tip (Schuster et al. 2012). Interestingly, the MMD domain and actin have been also proposed to facilitate a minor lateral delivery of Mcs1 protein, which could be functionally equivalent to the alternative delivery route described in *S. cerevisiae* (Valdivia et al. 2002). It remains to be tested whether this non-polarised delivery of Mcs1p is altered under stress conditions. However, this model does not address how some filamentous fungi could support chitin synthesis at the hyphal tip in the absence of class V/VII CSs. Information about this issue has been gained from studies in yeasts by comparing budding and hyperpolarised growth. During budding, Chs3p localisation at the neck relies on a delicate balance between anterograde delivery and endocytosis (Reyes et al. 2007). However, during hyperpolarised growth, the endocytic recycling of Chs3p is reduced, allowing Chs3p accumulation along the mating projections in *S. cerevisiae* or at the hyphal tip in *C. albicans* (Sacristan et al. 2012). Thus, the modulation of the endocytic recycling of class IV enzymes could suffice to switch the chitin synthesis programme from bud to hyphal growth. It is tempting to speculate about the possibility of a role for endocytosis in Mcs1p traffic, since endocytic recycling could replenish, at least partially,

the Mcs1p-containing pool of vesicles (see above). However, this hypothesis has not yet been tested.

VII. Chitin Synthesis and Antifungal Therapies

Fungal cell walls have traditionally been considered as an attractive target for antifungal therapies. Among the various cellular components, chitin emerged as an ideal target owing to its absence in plants and animals, both of which are hosts for fungal infections. This promise was somehow fulfilled after the initial discovery of Polyoxin D, a molecule with in vivo antifungal activity through the inhibition of chitin synthesis (Endo et al. 1970). Further work led to the identification of new polyoxins and the related family of nikkomycins, all of them acting as antifungal agents through competitive inhibition of chitin synthases [reviewed in Debono and Gordee (1994)]. Moreover, genetic analyses performed in *S. cerevisiae* revealed the synthetic lethality of the *chs2Δ chs3Δ* double mutant (Shaw et al. 1991), reinforcing the view of chitin as an essential component of the fungal cell wall and of chitin synthesis inhibition as an ideal antifungal target. Unfortunately, after decades of research in the field none of these compounds has attained therapeutic value owing to their poor efficacy in vivo. The causes of this failure are not fully understood, but two major reasons are apparent: (1) the poor bioavailability of the drugs due to inefficient transport systems (Debono and Gordee 1994), and (2) the presence of several CS isoenzymes in the cell with dramatic differences in the sensitivity to these compounds. This was demonstrated for Nikkomycin Z, which proved to be a specific inhibitor of *Sc*CSIII with null effects on *Sc*CSII, explaining the poor in vivo effect of this drug on the growth of *S. cerevisiae* (Gaughran et al. 1994). Similarly, RO-09-3143 has been identified as a specific inhibitor of CaCSII activity, showing only a fungistatic effect on *C. albicans* growth (Sudoh et al. 2000). In view of the diversity of the fungal CSs determined to date and the potential biological redundancy between them, the poor antifungal efficacy of polyosins/nikkomycins in vivo is not surprising. We must therefore await the identification of better chitin synthase inhibitors with broader specificities against the different classes of chitin synthases. Success in this will probably be linked to a better knowledge of the distinct regulation of different chitin synthases.

Interestingly, chitin synthesis not only depends on CS activity, but also on the metabolic synthesis of its substrate (UDP-NAcGln), a metabolic bottle neck that depends on the activity of Gfa1p (see above). This makes it an additional target for antifungal therapies (Bulik et al. 2003). In this line of work, inhibitors of *Af*Agm1p (*A. fumigatus N*-acetylphosphoglucosamine mutase, another enzyme involved in the synthesis of the substrate for CSs) have recently been identified (Fang et al. 2013), opening new perspectives for the future development of antifungal agents.

Recent evidence indicates that chitin synthesis has not only failed to hold its promise as an antifungal target, but indeed has become a problem in alternative antifungal therapies using β-glucan synthase inhibitors. The clinical use of caspofungin revealed the so-called paradoxical effect of this drug, which is less active against *C. albicans* in vivo at higher concentrations. This effect proved to be dependent on an increase in chitin synthesis that affects cell wall architecture, making cells more resistant to the drug (Munro 2013) and less accessible to immune recognition by the host (Marakalala et al. 2013).

After the initial perspectives and decades of work on the identification of inhibitors of chitin synthesis, it is now becoming accepted that no "magic bullet" is waiting in the wings. Instead, the solution for the treatment of fungal infections can be achieved by exploiting the synergistic effect of chitin synthase inhibitors and some alternative antifungal therapies. This strategy is now being implemented in some animal studies with good results (Clemons and Stevens 2006), making the future introduction of some of these chitin synthesis inhibitors into clinical therapeutics possible.

VIII. Concluding Remarks

The genomic era has provided an extraordinary thrust in the characterisation of the diversity of chitin synthases in fungi, but this progress has not been translated to the same extent in our understanding of the functions of the different chitin synthases. Accordingly, the promise of chitin as ideal antifungal target has not been fulfilled.

Nevertheless, the extensive molecular work carried out in *S. cerevisiae* has made chitin synthases a paradigm in studies on the intracellular traffic of proteins. Chs3p traffic has proved to be extremely complex, highlighting some poorly known aspects about the intracellular traffic of polytopic proteins, such as the relevance of the exomer and AP-1 in their recycling. Our knowledge about this transport is underexplored in fungi and it is expected that more exhaustive work in filamentous fungi could provide additional information about CS regulation in the near future. In addition, studies on *Sc*Chs2p have provided evidence on how yeast cells coordinate actomyosin ring contraction with primary septum formation, whose relevance would be important to understand extracellular matrix remodelling during eukaryotic cytokinesis. Thus, CS studies still remain an exciting field of research.

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