UNIVERSITÄT OSNABRÜCK

Proteolytic processing of fungal and insect proteins involved in chitin synthesis

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1 Introduction

1.1 Chitin

Chitin is one of the most widespread polysaccharides in nature. An estimated annual production of 10 gigatons makes chitin, after cellulose, the second abundant organic compound worldwide (Muzzarelli, 1999). The Name chitin originates from the Greek word *chiton* meaning tunic or envelope. Chitin is synthesized by a large variety of taxonomic groups. It can be found among other in bacteria, protozoa, arthropods, bivalves, and even vertebrates (bony fish) (Table 1). In these organisms chitin is an extracellular compound and functions as scaffolding material to stabilize cysts, cell walls, shells, cuticles or peritrophic matrices (Merzendorfer, 2009). Depending on its interactions with proteins or other molecules, its physical properties vary enormously being tough and rigid or ductile and elastic. The chitin microfibrils are embedded into extra cellular matrices in a way that resembles constructions of steel reinforced concrete (Gooday, 1990; Muzzarelli, 1977).

Organism	**Localization**	**Function**
Bacteria (Rhizobiacea)	Secreted, extracellular space	Nodulation of leguminous plant roots
Protozoa	Cyst	Physical and chemical resistance
Fungi	Cell Wall Spore Wall Septa	Compensation of turgor pressure (1.2.1.2.) Physical and chemical resistance Stabilization of cell division zones
Nematodes	Pharynx Egg Shell Gut peritrophic matrix	Mechanical breakdown of food Physical and chemical resistance Multiple protective functions
Arthropods	Epidermal cuticles, Shells Tracheal cuticle Gut peritrophic matrix Egg Shells	Cuticle differentiation, skeletal functions Tracheal development, skeletal functions Multiple protective functions Physical and chemical resistance,
Bivalves	Shell	Shell and nacre formation
Gastropods	Snail Shell, radula Gut peritrophic matrix	Shell formation, grazing Multiple protective functions
Cephalopods	Squid pen, cuttlefish bone Stomach cuticle	Endoskeleton Protective functions
Tunicates	Integument (test) Gut peritrophic matrix	Reinforcement Multiple protective functions
Vertebrates (bony fish)	Fin cuticle	Reinforcement

Table 1. *Functions of chitinous structures in various chitin-synthesizing organisms* - (modified after Merzendorfer, 2009).

Chitin is an unbranched sugar polymer consisting of *N*-acetylglucosamine residues (GlcNAc), which are linked by [1,4-β]-glycosidic bonds. Due to the properties of the [1,4-β]-glycosidic bonding, neighboring sugar monomers are rotated by 180° to each other (Figure 1). The proper repeating subunit is therefore the dimer chitobiose. Chitin exhibits great structural similarity to cellulose, which only differs in its sugar monomers being β-D-glucose in contrast to N-acetylated glucosamine. This allows stronger hydrogen bonding between neighboring polymers, making chitin more compact (Rudal and Kenchington, 1973). The lengths of chitin chains are estimated to be 5,000 to 10,000 GlcNAc residues in insect cuticles whereas in cell walls of fungi, depending on the species, the length varies significantly between 100 and 8,000 monomers (Cabib, 1987; Hackman *et al*., 1987; Kang *et al*., 1984; Muzzarelli *et al*., 1986). Individual chitin polymers form microfibrils with a diameter between three and ten nm and with lengths ranging between a few nm to 2 µm (Merz *et al*., 1999). Microfibrils can orient in a parallel (β), anti parallel (α) or alternating (γ) manner (Minke and Blackwell, 1978; Rudal and Kenchington, 1973). α-chitin is the most abundant and most solid form; it is found mainly in cell walls from yeasts or cuticules from arthropods and also in some mollusks and nematodes (Cohen, 1993; Muzzarelli, 1977). In β-chitin and γ-chitin the quantity of hydrogen bonds is reduced and therefore the multimers are less packed, which also leads to higher hydration. This more flexible chitin can be found in marine protozoa as well as in cocoons and peritrophic matrices of arthropods (Blackwell, 1969; Dweltz, 1961; Dweltz and Anand, 1961; Herth *et al*., 1986; Kenchington, 1976; Mulisch *et al*., 1983; Peters, 1992). Chitin is achromatic, odourless and basophile. It is insoluble in water and numerous other detergents like ethanol, ether, chloroform, acetone, ammonia, diluted acids or concentrated bases. In nature chitin is commonly enzymatically deacetylated. The rate of acetylation can vary; predominately deacetylated chitin is called chitosan (Muzzarelli *et al*., 1986).

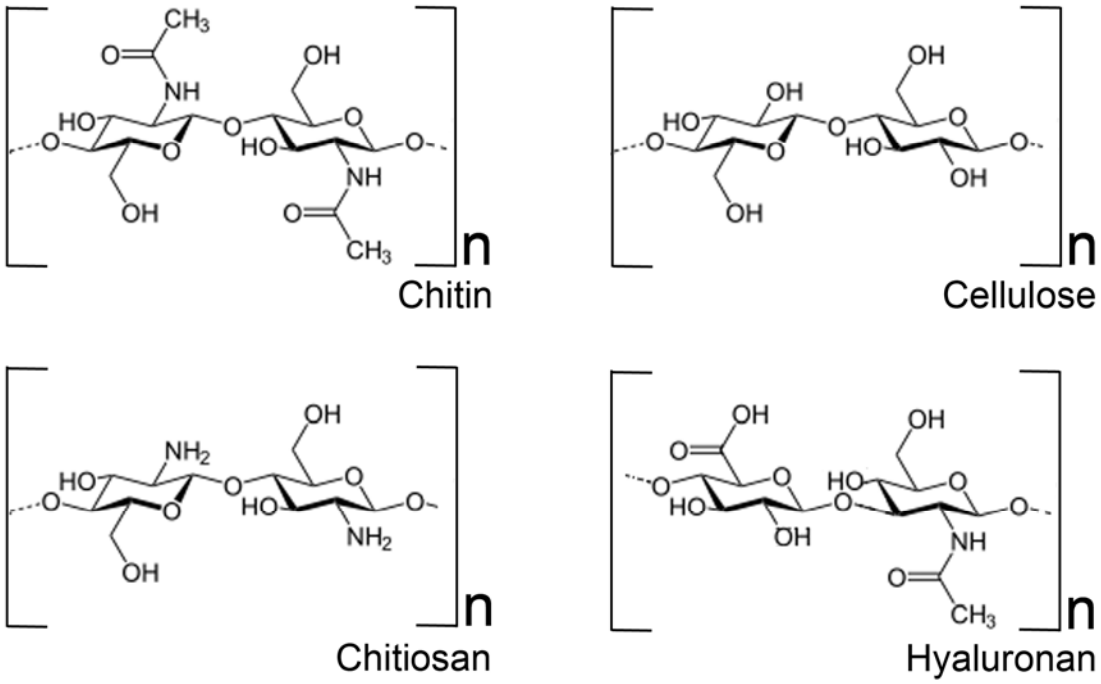


Figure 1. Chemical structure of chitin, cellulose, chitosan and hyaluronan – the repetitative units of chitin, cellulose, chitosan and hyaluronan are disaccharides indicated in brackets.

1.2 Chitin synthase

The obvious function of chitin synthases is the production of the biopolymer chitin. Chitin synthases belong to the family 2 of glycosyltransferases (GTF2), a large family of enzymes with members including cellulose synthases and hyaluronan synthases (Coutinho *et al*., 2003); see Figure1 for structure of polymers). The proteins in this family are inverting processive glycosyltransferases that form β-linkages. The activated sugar donor, containing a substituted phosphate group, is linked to the non-reducing end of the growing polymer. Chitin synthases catalyze the conversion of UDP-N-acetyl-D-glucosamine and (1,4-(N-acetyl-beta-D-glucosaminyl))(n) into UDP and (1,4-(N-acetyl-beta-D-glucosaminyl))(n+1) (Imai *et al*., 2003). These multispanning membrane proteins not only synthesize the chitin polymer but in addition are probably involved in the translocation of the nascent polymer across the membrane into the extracellular space. The amino acid similarity of chitin synthases differs considerably, however, seven conserved regions have been characterized, with most of them being directly or indirectly involved in catalysis:

UDP-binding (region 1: K-(β-fold)-DDGS and region 2: R-(β-fold)-GKR), donor saccharide-binding (region 3: hD(S/A)DT (also known as DXD-motif) and region 4: G(X)4(Y/F)R), acceptor saccharide-binding (region 5: GEDRXX(T/S)) and product-binding (region 6: (Q(Q/R)XRW). A seventh region ((S/T)WGT(R/K) motif) can be found outside the projected catalytic center (Figure 2; (Merzendorfer, 2006).

Three mayor issues still remain to be solved: The initiation of polymerization, sterical problems for substrate binding and the translocation of the polymer across the plasma membrane. The polymerization reaction of chitin synthases is probably initiated by a primer, which is either soluble or covalently bound to the enzyme (Heifetz *et al*., 1979; Horst, 1983; Mills and Cantino, 1980; Quesada-Allue, 1982). The [1,4-β]-linkage in chitin requires each GlcNAc residue to be flipped nearly 180° with respect to its neighbors.

There are three theoretical solutions for the enzyme to utilize a β-linkage: either the sugar chain or the synthase rotates continuously by 180° or the added sugar residues have to be rotated by a different sugar binding side of the enzyme into the proper orientation (Richmond, 2000). It has been shown, that related hyaluronan synthases, which in contrast to chitin synthases have to link two different sugar monomers sequentially with different β-linkage (1,3; 1,4), hold two sugar binding sites, supporting the latter solution (DeAngelis, 1999). Another solution however would be a reaction involving two different catalytic centers provided not by a single monomer but by a homodimer of two chitin synthases (Merzendorfer, 2006). Indeed, purification of MsChs2 from the larval midgut of *Manduca* revealed an oligomeric chitin synthase complex (Maue *et al*., 2009; Zimoch, 2007). Two hybrid analyses in yeast, identifying self interaction of the chitin synthase 3, also support oligmerisation of chitin synthases (DeMarini *et al*., 1997). Closely related plant cellulose synthases also oligomerize and form hexagonal structures, so-called rosettes consisting of 36 single units (Kimura *et al*., 1999; Richmond and Somerville, 2000). The oligomerization could also provide a solution for translocation of the polymer across the plasma membrane by forming pore like structures responsible for translocation of the nascent polymer (Merzendorfer, 2006). In addition the

(S/T)WGT(K/R) motif, found in the C-terminal part of chitin synthases, might be involved in translocation (Cohen, 2001; Merzendorfer, 2006).

1.2.1 Chitin synthases in insects

All known insect chitin synthases are multispanning membrane proteins with theoretical molecular masses of about 170 kDa. Based on amino acid analysis Tellam *et al*. (2000) suggested, a model, in which the central catalytic domain is flanked by two transmembrane domains (Figure 2). The N-terminal domains contain 7-10 transmembrane helices. In contrast to the N-terminal domain, the catalytic domain (about 400 amino acids) is highly conserved and characterized by 6 conserved regions involved in catalysis (Merzendorfer, 2006). Topological studies performed with other glycosyltransferases suggest that the catalytic domain is located in the cytoplasm. However, due to the lack of topological studies on chitin synthases, extracellular localization can not be excluded (Merzendorfer, 2006). The C-terminal domain contains seven membrane helices and may be involved in translocation of the nascent chitin polymer (Tellam *et al*., 2000).

Based on current knowledge, insects have only two genes encoding chitin synthases, Chs1 and Chs2 (also named Chs-A and Chs-B; Merzendorfer, 2006). While Chs1 is specialized on chitin synthesis in the epidermal and tracheal cuticle, Chs2 is responsible for chitin synthesis in the course of peritrophic matrix formation by midgut epithelial cells (Arakane *et al*., 2005; Hogenkamp *et al*., 2005; Lehane, 1997; Zimoch *et al*., 2005). Chs1 expression is up-regulated during molt and down-regulated during intermolt, whereas Chs2 expression is down-regulated during molt and up-regulated during the intermolt (Zimoch *et al*., 2005; Liang *et al*., 2010; Chen *et al*., 2007). In feeding larvae the chitin synthase 2 is localized at the very apical tips of the brush border microvilli formed by midgut columnar cells (Arakane *et al*., 2005; Zimoch and Merzendorfer, 2002). In addition to transcriptional regulation, it is postulated that chitin synthases are regulated posttranslationally by proteolytic activation. The enzymes are synthesized as zymogens and activated by trypsin or other serine peptidases (Duran and Cabib, 1978; Merz *et al*., 1999; Merzendorfer, 2006; Roncero, 2002). The zymogenic character of chitin synthases has been

extensively studied in the course of the past 30 years. However, up to now, no proteases with the capability to activate chitin synthesis *in vivo* could be identified. Broehan *et al*. (2007) described the first protease interacting with a chitin synthase. A yeast two hybrid screening using the C-terminal, extracellular subdomain C7 of MsChs2 as bait identified an interaction with a chymotrypsin like protein (MsCTLP1). This interaction was confirmed by co-immunoprecipitation and colocalization studies.

By monitoring the immunoreactivity with antibodies as well as enzymatic activities of the respective samples, a purification protocol for the chitin synthase 2 complex from the *M. sexta* midgut could be established. The molecular mass of the active enzyme determined by gel permeation chromatography and Native-PAGE is about 520 kDa, thrice the molecular mass of the chitin synthase monomer (Maue *et al*., 2009). In line with the suggested zymogenic nature of the chitin synthase, gelelectrophoresis of the 520 kDa complex under denaturing conditions revealed a protein pattern that can be explained by two cleavage events.

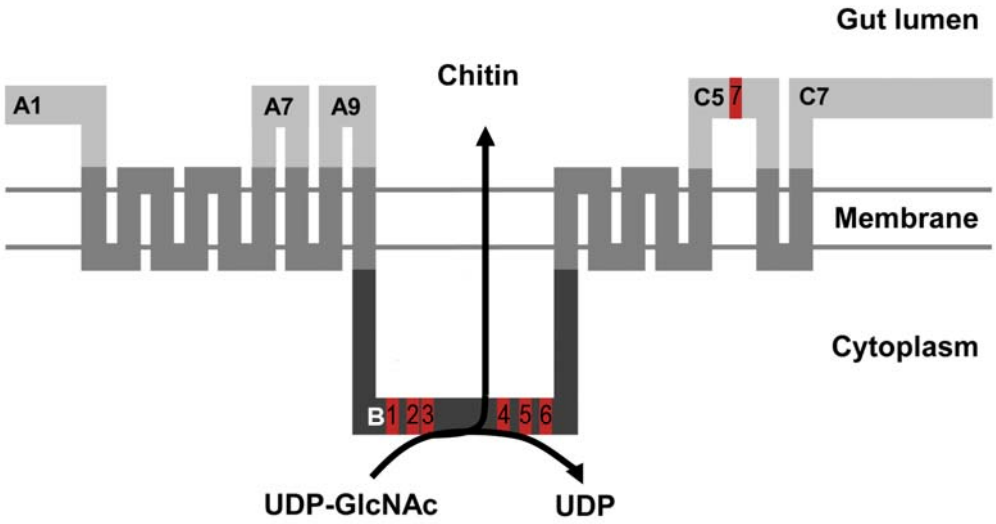


Figure 2. Domain architecture of the midgut chitin synthase MsChs2 - The two horizontal lines represent the apical brush border membrane of the midgut columnar cells. Horizontal bars represent intra- or extracellular domains and vertical bars transmembrane helices. (A) N-terminal transmembrane region; (B) cytoplasmic catalytic domain; (C) C-terminal transmembrane region; the numbers refer to single domains within each region. Regions 1 and 2 UDP-binding; regions 3 and 4 donor saccharide-binding; Region 5 acceptor saccharide-binding; region 6 product-binding; region 7 possibly involved in chitin translocation; (modified after Merzendorfer, 2006 and Broehan *et al*., 2007).

1.2.2 Chitin synthesis in fungi

Fungal chitin synthases (Chs) are much more diverse than their relatives in insects. Amino acid sequence alignments of fungal chitin synthases reveal that the fungal proteins can be classified into 2 divisions that branch into seven classes (Martin-Urdiroz *et al*., 2008; Roncero, 2002; Ruiz-Herrera *et al*., 2002). Division 1 includes classes I-III, were as division 2 is subdivided into classes IV, V, VI, VII and chitin synthase from animals (Figure 3). Chitin synthases of classes I, II and IV are found in yeasts and filamentous fungi.

The baker´s yeast *Saccharomyces cerevisiae* has 3 different chitin synthases, Chs1-3. The yeast Chs1 and Chs2 are members of the classes I and II, respectively (Ruiz-Herrera *et al*., 2002). Class IV contains Chs3, being the closest related *S. cerevisiae* chitin synthase to insect chitin synthases. In contrast to *S. cerevisiae* the human pathogen *Candida albicans* has four chitin synthase isoenzymes (CaChs1, CaChs2, CaChs3, and CaChs8, Munro *et al*., 2003). CaChs1 is grouped in class II and is homolog to Chs2 while CaChs2 is the *C. albicans* homolog to Chs1 and is a member of the class I. CaChs8 is also a class I chitin synthase enzyme and makes a minor contribution to chitin synthase activity measured *in vitro.* CaChs3 is closely related to Chs3 and grouped in class IV (Munro *et al*., 2007; Munro *et al*., 2003).

Class III, V, VI and VII chitin synthases can only be found in filamentous fungi. Filamentous fungi species exhibit chitin synthases of the classes V, VI and VII and contain a myosin motor-like domain which presumably has a function in the actin-based localization of these proteins (Martin-Urdiroz *et al*., 2008). Class V chitin synthases carry large myosin motor-like domains, while classes VI and VII synthases carry shorter myosin motor-like domains lacking an ATP-binding site. Interestingly, the chitin synthase from the mollusks *Mytilus galloprovincialis* exhibits a similar domain structure. This chitin synthase also contains a myosin motor head domain and is expressed specifically in the shell forming tissue of the mollusk larvae (Weiss *et al*., 2006).

The filamentous mold fungi *Aspergillus nidulans* carries at least 7 different chitin synthase genes *AnCHSA*, *AnCHSB*, *AnCHSC*, *AnCHSD, AnCHSE*, *AnCSMA, AnCSMB* and *An4367.2* (one is coding for a hypothetical protein). Their respective gene products belong to the classes II, III, I, IV, V and VI,

respectively (Tsuizaki *et al*., 2009); Figure 3); where as in *Aspergillus fumigatus* eight different *CHS* genes have been identified with at least one gene product in every fungal class (Figure 3).

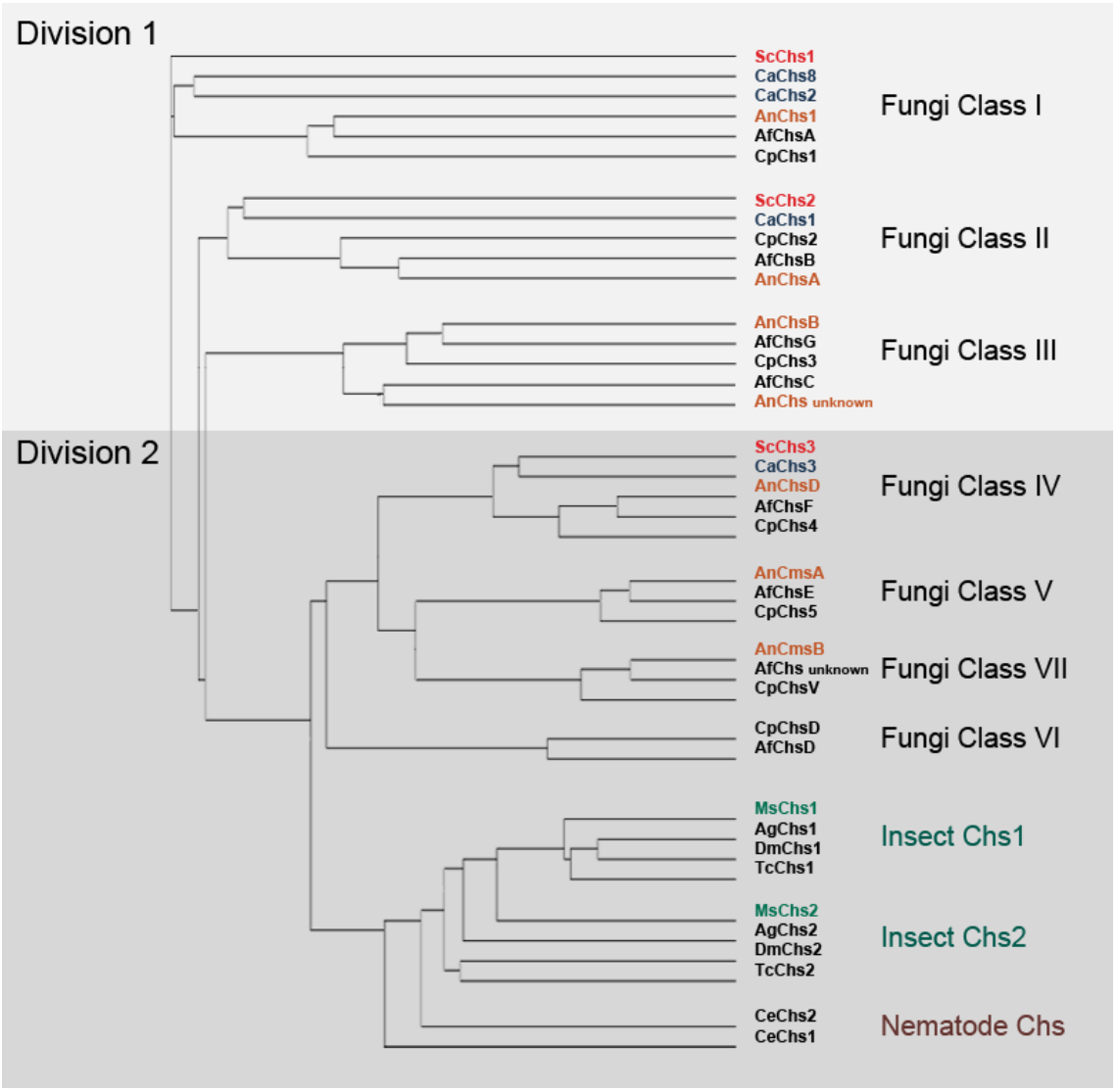


Figure 3. ClustalW cladogram showing phylogenetic classes of fungi and animal chitin synthases - The tree is based on a ClustalW alignment (Thompson *et al*., 1994). The following sequences were used in the alignment: Chs1-3 (*Saccharomyces cerevisiae*),CaChs1-3 and CaChs8 (*Candida albicans*), AnChs1, AnChsA, AnChsB, AnChsD, AnCmsA, AnCmsB, AnChs unknown (*Aspergillus nidulans*), AfChsA-G, AfChs unknown (*Aspergillus fumigatus*), CpChs1-5, CpChsV (Coccidioides posadasii), AgCHS1-2 (*Anopheles gambiae*), DmCHS1-2 (*Drosophila melanogaster*), MsCHS1-2 (*M. sexta*), TcCHS1-2 (*Tribolium castaneum*), CeCHS1-2 (*Caenorhabditis elegans*). Proteins discussed in the thesis are highlighted in different colors (modified after Ruiz-Herrera *et al*., 2002 and Martin-Urdiroz *et al*., 2008).

1.2.2.1 Cell wall construction in yeast

Most cells exhibit an extracellular matrix surrounding the plasma membrane. The composition of extracellular matrices differs significantly between taxonomic groups. The extracellular matrices of bacteria, archaea, plants and fungi are thick meshworks composed of polysaccharides and proteins and are termed cell walls. In contrast to that, the fungal cell wall mainly consists of glucan, mannoproteins and to a lesser degree of chitin (Klis *et al*., 2006; Lesage and Bussey, 2006). In yeast the cell wall has four major functions; stabilization of internal osmotic conditions, protection against physical stress, maintenance of cell shape and finally the cell wall as a scaffold for proteins (Klis *et al*., 2006). The baker yeast cell wall makes up to 25% of the dry weight of the total cell mass (Aguilar-Uscanga and Francois, 2003). It is a complex network, which is, despite its durability, extendible. Under hypertonic conditions, the yeast cell rapidly shrinks and loses up to 60% of its volume, reducing the surface of the cell wall up to 50%. This process is fully reversible, if cells are subsequently transferred into isotonic media (Morris *et al*., 1986). Depending on growth conditions the cell wall consists of 35-55% glucan, 30-50% mannoproteins and 1.5 – 6.0% of chitin. Electron microscopical studies of vegetative cell walls identified an inner layer of glucans and chitin and an outer layer of mannoproteins (Osumi, 1998).

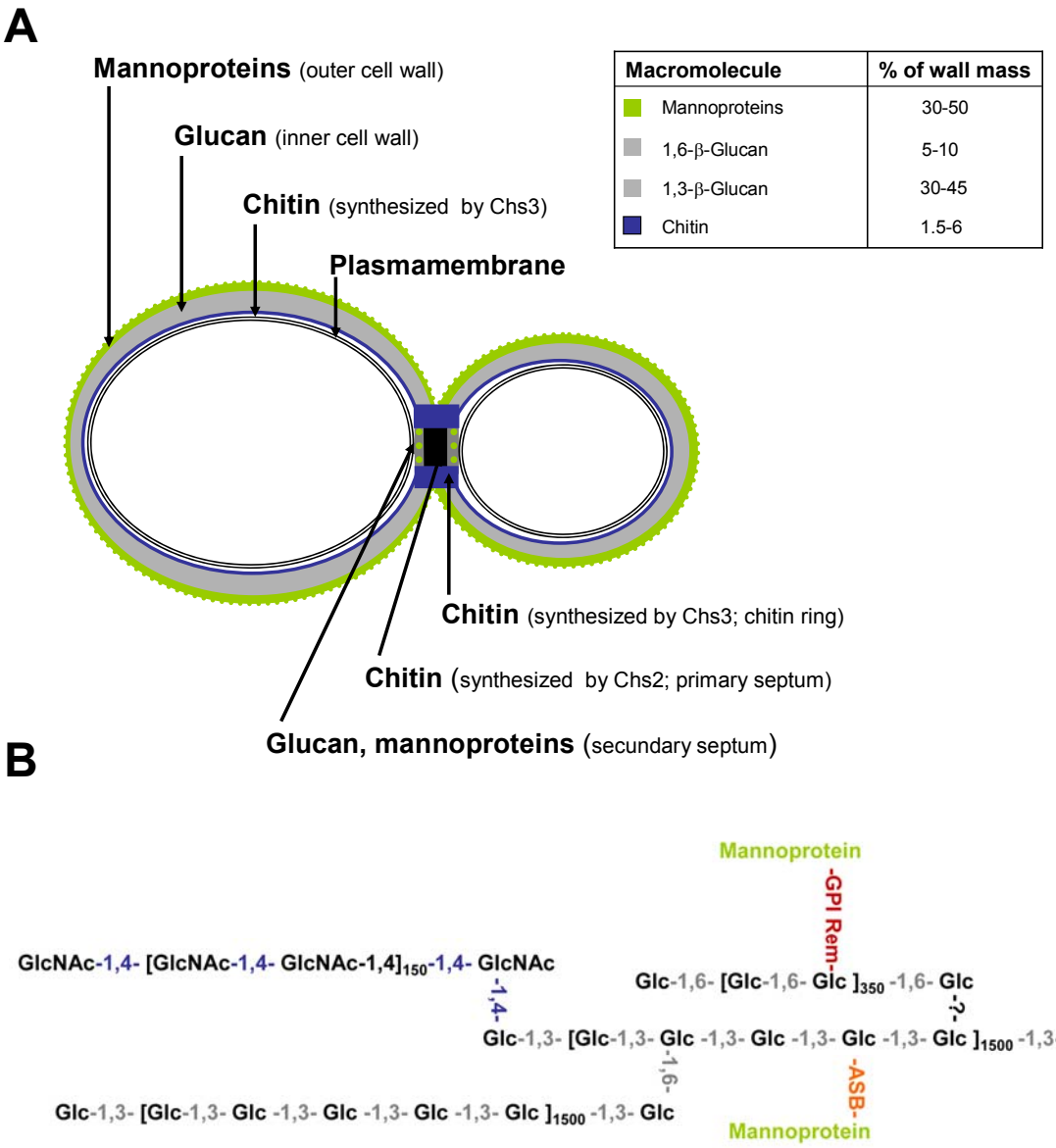


Figure 4. Composition of the cell wall of S. cerevisiae – (**A**) The cell wall components are presented in the order in which they are found *in vivo* from the outside to the inside. Chitin is synthesized by three different chitin synthases. Chs3 synthesizes the chitin in the lateral cell wall and in the chitin ring were as Chs2 synthesizes the chitin in the primary septum. Chs1 only functions as a repair protein and contributes minor to chitin abundance in the cell wall. Cell wall stress may lead to dramatically increased chitin levels. (Table modified after Klis *et al.*, 2006) (**B**) The [1,3-β]-, [1,6-β]- and [1,4-β]-glucosidic bonds are indicated in grey and blue, respectively. Cell wall mannoproteins can be linked to the [1,3-β]-glucan via alkali-sensitive bonds (ASB) or to GPI-anchored cell wall mannoproteins. (GPI Rem.). The links between [1,3-β]-glucan and [1,6-β]-glucan are still uncharacterized (shown as ?); (modified after Lesage and Bussey, 2006).

The mayor cell wall component is glucan, which is a polysaccharide of D-glucose monomers linked either by [1,3-β]- or [1,6-β]-glycosidic bonds. The [1,3-β]-glucan alone contributes to 30-45 % of the wall mass. The [1,3-β]-

glucan chains consist of about 1,500 sugar monomers, which are branched ([1,6-β]-interchain links). The polymer is synthesized by the [1,3-β]-D-glucan synthase complex. This complex consists of a regulatory subunit Rho1 and a catalytic subunit, which is encoded by FKS1 and GSC2 (Douglas *et al*., 1997; Klis *et al*., 2006). In *S. cerevisiae* [1,6-β]-glucan has an amorphous structure and its chains are much shorter than [1,3-β]-polymers. The [1,6-β]-glucan acts as a flexible glue by forming covalent cross-links to [1,3-β]-glucan, mannoproteins and supposedly chitin (Kollar *et al*., 1997; Figure 5).

Mannoproteins form an electron-dense, fibrillar outer layer of the cell wall. Mannan modifications of protein are anchors either by *N*- or *O*-mannosyl bonds (Figure 4; Wilson *et al*., 2009) *O*-linked glycans consist of up to five mannose monomers, with the first two residues being [1,2-α]-linked and the subsequent ones [1,3-α]-linked. In *S. cerevisiae* cell wall proteins are mannoglycosylated in a two step manner. The first step occurs in the endoplasmic reticulum (ER). Comparable to many other intracellular proteins, 9 to 13 mannose residues are linked N-terminally to the proteins (“core”-type). In a second step, which occurs in the Golgi, up to 50 mannose monomers are linked to the core mannose residues (Lesage and Bussey, 2006; Wilson *et al*., 2009; Figure 4). In addition to *N*-glycosylation and/or *O*-glycosylation the majority of cell wall proteins receive a glycosylphosphatidylinositol (GPI) anchor during their passage through the secretory pathway. The addition of a GPI anchor takes place at the ER and substitutes a hydrophobic domain at the C-terminus of the proteins. Outside the cell, the cell wall proteins, become attached to [1,6-β]-glucan chains through a remnant of the GPI anchor (Kollar *et al*., 1997).

Although chitin is only a minor constituent of the yeast cell wall (~2% of dry weight), its synthesis is essential during cell division and sporulation. During cell division, chitin is initially concentrated in a ring-like structure at the site where the bud emerges, and then deposited as a disc forming the primary septum (Lesage and Bussey, 2006). Up to 50% of the chitin polymers are linked at reducing ends to the non-reducing end of [1,3-β]-glucan via [1,4-α]-bond, stabilizing the cell wall network (Lesage and Bussey, 2006; Figure 4).

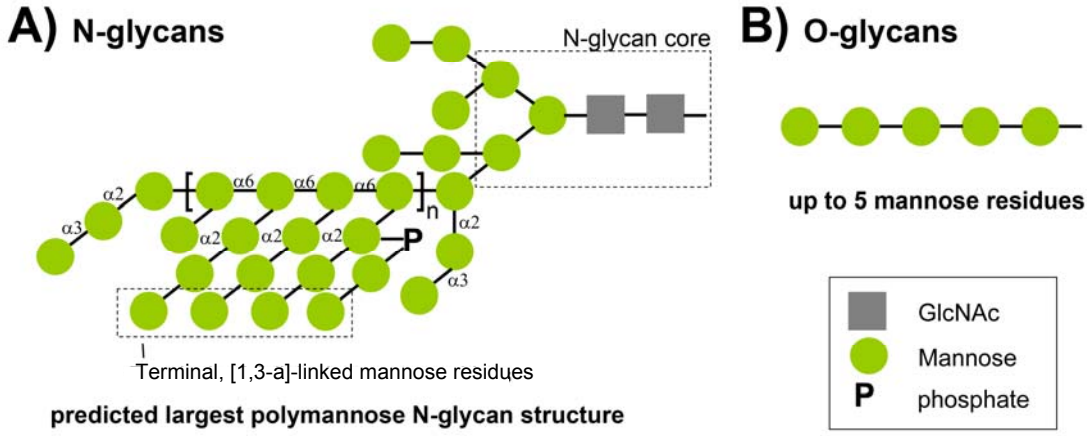


Figure 5. Gycosylation in *S. cerevisiae* – (**A**) Yeast species add more mannose residues to their *N*-glycans than other species. (**B**) *O*-gylcolisation in *S. cerevisiae* (modified after Wilson *et al*., 2009).

1.2.2.2 Chitin synthesis in vegetative yeast cells

Chitin synthesis in baker yeast is catalyzed by three membrane-integral chitin synthases encoded by the genes *CHS1*, *CHS2* and *CHS3* (Cabib *et al*., 1993). Although the three enzymes can be activated by proteolytic treatment *in vitro*, it is still unclear, if yeast chitin synthases are synthesized as precursors *in vivo* and if they are subsequently converted from zymogens into catalytically active forms (Cos *et al*., 1998; Lesage and Bussey, 2006). Chs1 acts as a repair enzyme which is involved in remodeling of the cell wall during cell division. Most of the protein is present in chitosomes and a minor fraction localizes to the plasma membrane (Leal-Morales *et al*., 1994). Chs2 is transported through the secretory pathway to the plasma membrane at the bud neck (Chuang and Schekman, 1996) and synthesizes chitin in the central disc of the primary septum. Chitin produced by Chs2 is largely unbound to the cell wall network (Klis *et al*., 2006). A recent study revealed that Chs2 is phosphorylated. In heterologous expressed Chs2 twelve N-terminal phosphorylation sites were identified (Martinez-Rucobo *et al*., 2009). Moreover Martinez-Rucobo *et al*.(2009) demonstrated that Chs2 is proteolytic processed by a presently unknown protease, which is expressed during exponential growth phase.

Chs3 accounts for about 90% of the chitin produced *in vivo* (Shaw *et al*., 1991), and is required for chitin synthesis at the lateral cell wall in young daughter cells and later in young mother cells for the formation of the chitin ring at the bud

neck (Cabib *et al*., 1996). The chitin ring is anchored to the existing glucan network by attachment to [1,3-β]-glucan chains (Cabib and Duran, 2005). The precise number and topology of transmembrane helices and soluble domains of Chs3 are uncertain. Fluorescence microscopy studies revealed that Chs3 localizes in smaller amounts to the plasma membrane, in higher amounts to the membranes of post-Golgi vesicles, and during cell division to a ring-like structure at the bud neck of small-budded cells (Cabib *et al*., 1993).

Chs3 is synthesized by ribosomes and inserted into the lipid bilayer of the ER, where it eventually attains its native conformation. This process requires the presence of the ER chaperone Chs7, which seems to prevent Chs3 aggregation (Trilla *et al*., 1999). After processing of Chs3 in the ER and Golgi-apparatus, including glycosylation and palmitoylation, it is transported from the trans-Golgi network to the cell surface, a process that requires Chs5 and Chs6, which are part of an exomer coat-complex (Lam *et al*., 2006; Santos and Snyder, 1997; Wang *et al*., 2006).

At the bud neck Chs3 is linked to septins via Chs4 and Bni4 (DeMarini *et al*., 1997). Bni4 also recruits the catalytic subunit of protein phosphatase 1 (Glc7) to the bud neck in a temporal and spatial restricted manner, a process that assists in recruiting Chs3 by a yet unidentified substrate (Larson *et al*., 2008). Like Chs1, Chs3 is not degraded in vacuoles, but accumulates in chitosomes, which are specific secretory vesicles for chitin synthase transport to the plasma membrane (Ruiz-Herrera *et al*., 1977). They appear to act also as a trans-Golgi reservoir that is replenished by the endocytotic turnover of the enzyme (Ziman *et al*., 1996). Under conditions of cell wall stress, such as absence of other cell wall component proteins or exposure to cell surface-perturbing agents, Chs3 activity is increased up to 5- fold leading to significantly elevated levels of chitin in the cell wall (Bulik *et al*., 2003).

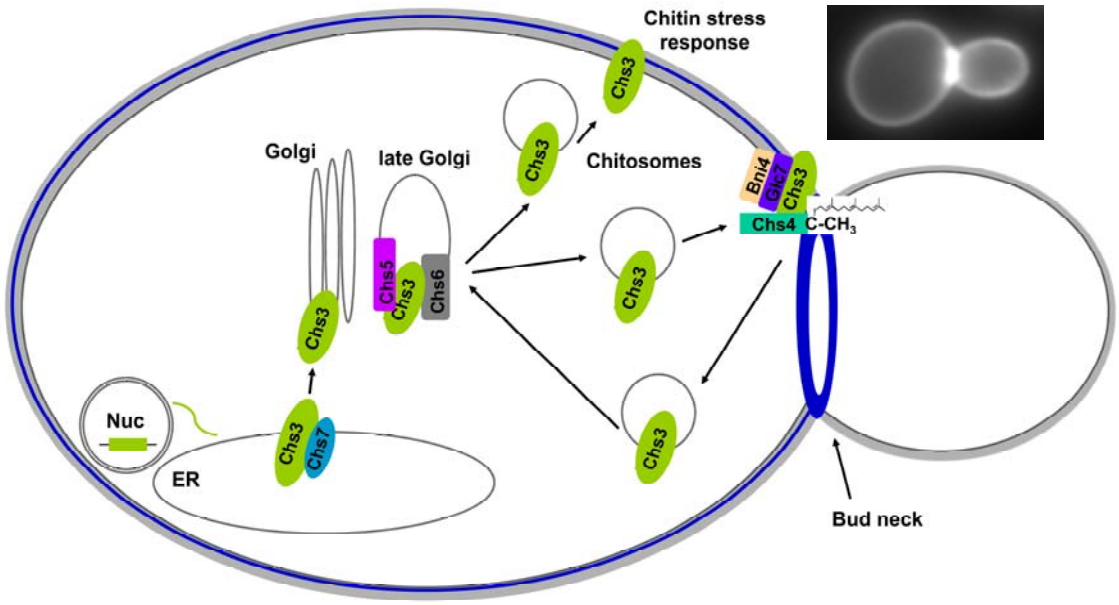


Figure 6. Trafficking and biosynthetic pathways of Chs3 - The CHS3 gene and its products are represented in red. The chitin made by Chs3 is shown in dark blue. The cell wall, the plasma membrane, and chitin are shown in gray and black, nucleus, Nuc; vacuole, Vac (modified after Lesage and Bussey, 2006). The micrograph shows the chitin deposition in *S. cerevisiae.* Cells were grown overnight in liquid YPD medium and stained with 0.02 % CFW.

1.2.2.3 Chitin synthesis in non vegetative yeast cells

In addition to vegetative growth, chitin is also synthesized during sporulation and mating (mating is described in the next paragraph). During sporulation the diploid cell converts into asci containing four haploid ascospores. The spore wall consists of four layers, the second one made of chitosan, a deacetylated derivative of chitin (Cid *et al*., 1995). Chitin is enzymatically deacetylated by sporulation-specific genes *CDA1* and *CDA2* (Lesage and Bussey, 2006). Interestingly, Chs3 activation has a dedicated activation pathway in ascospore synthesis, since *CHS4* is not required during sporulation. Chs3 activity can be stimulated by the sporulation specific Chs4 homologue Shc1 (Bulik *et al*., 2003; Cid *et al*., 1995; Lesage and Bussey, 2006).

In addition to sporulation, chitin is also synthesized during mating (Santos and Snyder, 1997). Chitin deposition occurs at the subapical portion of the shmoo tip (Lipke *et al*., 1976). *CHS1* transcription is highly induced by pheromone treatment whereas *CHS3* transcription is not. Still Chs3 activity is the activity responsible for the synthesis of chitin during mating, which leads to a 4-fold increase in chitin levels compared to vegetative cells (Bulik *et al*., 2003; Santos

et al., 1997). Deletion of *chs3* results in the loss of chitin in shmoos after treatment with mating pheromones, still *chs3*Δ mutants exhibit moderate mating efficiency (Roncero *et al*., 1988). By contrast *chs4*Δ and *chs5*Δ mutants are able to induce some chitin synthesis in response to pheromone, but in *chs5*Δ mutants the mating efficiency is drastically reduced (Bulik *et al*., 2003). Thus, in addition to its function in chitin synthesis, Chs5 plays a significant role in mating (Bulik *et al*., 2003).

1.2.2.4 Mating in yeast

Unlike most other microorganisms, the baker yeast *S. cerevisiae* has both a stable haploid and diploid state. Both, haploid and diploid yeast cells reproduce by mitosis. Haploid yeast cells have two mating types, **a** and α (Casselton and Olesnicky, 1998). *MAT***a** haploids express the genes **a**1 and **a**2 from the *MAT* locus while *MAT*α haploids express α1 and α2 from MAT (Haber, 1998). Wild-type yeasts are able to perform a mating switch by expressing the HO DNA endonuclease, which induces gene conversion, replacing the MAT**a** locus with the silent locus HMLα or the MATα with HMR**a,** respectively. Most laboratory strains are heterothallic, with stable mating types (Botstein, 2004). Haploid cells express a mating-type-specific peptide pheromone and the corresponding receptor.

Thus, **a** cells produce **a**-factors (pheromone) and the α-factor receptor (STE2), while α cells produce α-factors and the **a**-factor receptor (STE3; Casselton and Olesnicky, 1998). Induced by binding of the α-factor at *MAT***a** cells α-factor receptor and a-factor with *MAT*α cells **a**-factor receptor, cells go into vegetative arrest and become elongated and form shmoos toward their mating partner. This eventually leads to cell fusion of two haploid cells (Casselton and Olesnicky, 1998; Chen *et al*., 1997). Like many peptide hormones, the yeast mating pheromones are posttranslationally modified and proteolytically processed. While α-factors are secreted by the “classical” secretory pathway (ER→ Golgi→ secretory vesicles; Julius *et al*., 1984), **a**-factors are secreted by a “nonclassical” export mechanism (Michaelis, 1993). The peptide **a**-factor is initially synthesized as a precursor encoded by the functionally redundant

genes, *MFA1* and *MFA2* (Tam *et al*., 1998). The **a**-factor is sequentially processed, cleaved and exported by serials of proteins (Figure 7).

1.2.2.5 CaaX processing

CaaX processing includes a series of eukaryotic posttranslational modifications to the CaaX motif at the C-terminus of proteins. These posttranslational modifications increase the hydrophobicity of the proteins which results in membrane association of the modified proteins (Tam *et al*., 1998). The CaaX motif consists of four amino acids; a cysteine, two aliphatic amino acids (aa) and one non-conserved amino acid (X). The CaaX processing proteins undergo three sequential enzymatic modifications essential to their targeting: first, the proteins are prenylated by a covalent attachment of the cysteine in the CaaX motif to an isoprenoid lipid either farnesyl (C-15) or geranylgeraniol (C-20). The CaaX sequence is a substrate for farnesyl transferase if the X is one of the following amino acids: S, M, A, or Q. If X is a lysine or phenylalanine, the protein becomes a substrate for geranylgeranyl transferase I (Trueblood *et al*., 2000). In a second enzymatic step the last three amino acids of the protein (aaX) are proteolytically cleaved off by one of the two known CaaX proteases Ste24 or Rce1 depending on the aliphatic residues of the CaaX motif (Trueblood *et al*., 2000). Recently, however, it was shown that proteolytic processing of certain CaaX motifs can occur in the absence of the Rce1 and Ste24 proteases if the aliphatic residues are exchanged by polar amino acids (Krishnankutty *et al*., 2009). Finally, the newly exposed carboxylated group of the isoprenylcysteine is methylated. Prenylation plays an essential role in various cellular functions. CaaX proteins include nuclear lamins, Ras and a multitude of GTP-binding proteins (G proteins), several protein kinases, phosphatases and the yeast mating **a**-factor.

The **a**-factor is processed as followed (Tam *et al*., 1998): at first the CaaX farnesyltransferase complex Ram1/Ram2 transfers a farnesyl residue to the cysteine of the C-terminal CaaX sequence of the a-factor (CVIA). In a second step the C-terminal 3 aa –VIA are cleaved off by the CaaX-proteases Rce1 and Ste24, respectively. Then the new terminally prenylated cysteine is methylated by the methyltransferase Ste14. In addition to modifications at the C-terminus,

the N-terminus of the mating pheromone is also processed. Initially the first seven amino acids are cleaved off by Ste24, then the following 14 residues are cleaved by the metalloprotease Axl1 or its homologue Ste23 (Figure 7). Finally the mature, prenylated, carboxyl-methylated dodecamer is actively exported by the ABC-transporter Ste6 (Tam *et al.*, 1998). The question arises why an erstwhile soluble polypeptide is not secreted through the "classical" secretory pathway but instead prenylated, making the polypeptide extremely hydrophobic, and actively transported by an **a**-factor specific ABC-transporter. By prenylating the pheromone, diffusion in aqueous environments is decreased. This posttranslational modification may be critical in hydrophobic milieus, such as cellular membranes or natural habitats including biofilms and may also contribute to sharpening (increasing the steepness) or stabilizing of its gradient, avoiding unwanted induction of more distant α–cells (Arkowitz, 2009).

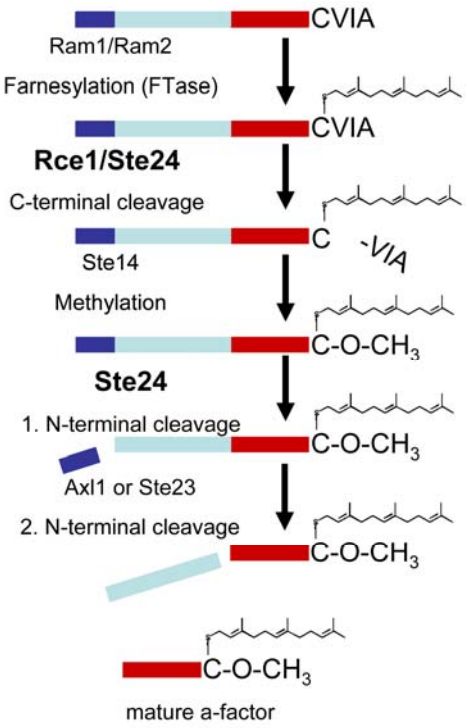


Figure 7. Pathway of a-factor biogenesis - The **a**-factor precursor, biosynthetic intermediates and components of the biogenesis machinery are shown. Note the dual roles of Ste24 (C-terminal aaX-ing and the N-terminal processing step; modified after Tam *et al.*, 2001).

1.2.2.6 Function and structure of Ste24

As described in the last paragraph Ste24 exhibits a dual role in a-factor processing. While it cleaves off the –VIA at the C-terminus in a prenylation dependent matter, it also is responsible for cleavage of the first six aa at the N-terminus, making it a unique protease (Tam *et al*., 1998). The zinc-dependent metalloprotease Ste24 is a membrane-resident protein with 7 transmembrane helices located in the ER membrane (Tam *et al*., 2001). Ste24 exhibits the characteristic catalytic metalloprotease motif HEXXH and in addition the C-terminal ER-retrieval dilysine motif (KKXX; Tam *et al*., 2001). Analyses of mutant cells expressing different **a**-factor CaaX variants demonstrated that Ste24 and Rce1, which also process the CaaX terminus of the **a**-factor (1.2.2.5.), have distinct but overlapping CaaX sequence specificities (Trueblood *et al*., 2000). Analysis of the *S. cerevisiae* genome revealed 98 proteins carrying CaaX motifs at the C-terminus; 35 of which are predicted to be a putative substrate of Ste24. Still until today the mating factor **a** remains the only characterized substrate. Ste24 homologues can be found in animals and plants (Bracha *et al*., 2002; Tam *et al*., 1998). It has been shown that the human homologue (ZmpSte24) catalyzes the proteolytic cleavage of C-terminal residues of farnesylated prelamin A to form mature lamin A, analogous to the cleavage of the **a**-factor in yeast. Interestingly, ZmpSte24 not only cleaves the farnesylated prelamin A at the C-terminal, but in addition also the N-terminus, similar to the **a-**factor cleavage by Ste24 (Figure 7). The mature intermediary filament lamin A stabilizes the nucleus of higher eukaryotes. Mutations in this gene have been associated with progeroid syndromes, mandibuloacral dysplasia and restrictive dermopathy (Young *et al*., 2005) .

1.3 Aims of the thesis

Chitin and chitin synthesis in particular has been extensively studied in insects and yeast for the last decades. Gene and cDNA sequences coding for chitin synthases were identified and characterized. However, many questions are still unsolved. In previous PhD theses from our lab, the first protease interacting with a chitin synthase *in vivo* was identified (Zimoch, 2007; Broehan *et al*., 2007). Moreover a protocol to purify active chitin synthase 2 form *M. sexta* midgut was established (Zimoch, 2007).

In order to extend current knowledge on Chs function and regulation, I have focused on the zymogenic nature of chitin synthases. In *S. cerevisiae*, the chitin synthase 3, which *in vivo* synthesizes the vast majority of cell wall chitin, is one of the best characterized chitin synthases and its intracellular trafficking has been studied in greater detail. Moreover, regulatory genes were identified and the zymogenic character has been characterized *in vitro*. However, until now, no protease has been identified which cleaves the zymogenic form of Chs3 *in vivo*.

Therefore, the main aim of my thesis was to identify proteases that are involved in proteolytic stimulation of chitin synthesis. In the beginning of this thesis, only limited information regarding *in vivo* proteolytic activation of chitin synthesis in yeast was available. Some data could be inferred from genome-wide studies in *S. cerevisiae*. A large-scale split ubiquitin screening by Miller *et al.* (2005) to identify inteactions between membrane proteins found among many others an interaction between Chs3 and Ste24. The aim was to confirm and to map the interaction between Chs3 and Ste24 and to investigate the influence of Ste24 on chitin synthesis in *S. cerevisiae* including intracellular localization of Chs3.

2 Material and Methods

2.1 Material

2.1.1 Chemicals, Enzymes, Kits and Reagents

Chemicals were purchased from Sigma-Aldrich Chemie GmbH (Munich, Germany), Invitrogen (Carlsbad, USA), Merck (Darmstadt, Germany), Qiagen (Hilden, Germany), Roche (Basel, Switzerland), Clontech (St-Germain-en-Laye, France), Stratagene (Cedar Creek, USA), NEB (Frankfurt a.M., Germany) and AbD Serotec (Duesseldorf, Germany) if not stated otherwise. Kits were used according to the manufacturer's protocols. Primers (Table 7) were synthesized at Eurofins MWG Operon (Ebersberg, Germany). Sequencing was performed by Sequencing Laboratories (Göttingen, Germany). Reagents used for production of media were obtained from Bacto Laboratories Pty. Ltd. (Mt Pritchard, NSW, Australia). All plasticware (reaction tubes, pipette tips, etc) were purchased from Sarsted (Nümbrecht, Germany).

2.1.2 Antibodies

Applied antibodies:

Antibody	conjugate	host	dilution	manufacturer
anti-CHS	-	rabbit	1:10000	(Zimoch and Merzendorfer, 2002)
anti-Myc	-	mouse	1:100	AbD Serotec,
anti-rabbit-IgG	alkaline phosphatase	goat	1:10000	Sigma
anti mouse-IgG	alkaline phosphatase	goat	1:30000	Sigma

Table 2. Antibody used in this work

2.1.3 Media

LB-Media	1% (w/v) tryptone , 0.5% (w/v) yeast extract, 0.5% (w/v) NaCl
ψb-Media	0.25% (w/v) yeast extract, 1% (w/v) peptone, 0.25% (w/v) $MgCl_2$ $7H_2O$ pH 7.6
YPD(A)-Media	2% (w/v) peptone, 1% (w/v) yeast extract, 2% (w/v) glucose, (1 g/L L-adenine)
YPDS-Media	YPD media,1M sorbitol
YPG-Media	2% (w/v) peptone, 1% (w/v) yeast extract, 2% (w/v) galactose
Drop-out-solution(10x)	0.2 g/L L-adenine hemisulfate, 0.2 g/L L-arginine HCl, 0.2 g/L histidine HCl, 0.3 g/L L-isoleucine, 0.3 g/L L-lysine HCl, 1 g/L L-leucine, 0.2 g/L L-methionine, 0.5 g/L L-phenylalanine, 2 g/L L-threonine, 0.2 g/L L-tryptophane, 0.3 g/L L-tyrosine, 0.2 g/L L-uracile, 1.5 g/L L-valine
SD-Media	0.67% (w/v) yeast extract without amino acids, 2% (w/v) glucose , 10% (v/v) drop-out-solution pH 6.5
LB- Plates	LB-media + 1.5% (w/v) agar
YPD(A)- Plates	YPD(A)-media, 2% (w/v) agar
SD -Plates	SD-media, 1.5% (w/v) agar
YPD^{CFW}-Plates	YPD plates, 50 µg/ml

Table 3. Media used in this work

2.1.4 *Escherichia coli strains*

Bacteria strains	**Genotyp**	**Referenz**
E. coli - K12 DH5α	F^-, Φ80d*lac*ZΔM15, Δ(*lac*ZYA-argF)U169, endA1, recA1, hs-dR17 ($r^-_K m^+_K$), deoR, thi-1, supE44, λ-gyrA96, relA1	(Hanahan, 1983)

Table 4. Bacteria strains used in this work

2.1.5 *S. cerevisiae* strains

Yeast strains	Genotype/description	Source or reference
BY4741	*MAT*a *his3*Δ*1 leu2*Δ*0 met15*Δ*0 ura3*Δ*0*	Euroscarf (Winzeler *et al*., 1999)
BY4742	*MAT*α *his3*Δ*1 leu2*Δ*0 lys2*Δ*0 ura3*Δ*0*	Euroscarf (Winzeler *et al*., 1999)
BY4741 *chs3*Δ	As for BY4741, *chs3*Δ::*kanMX4*	Euroscarf (Winzeler *et al*., 1999)
BY4741 *ste24*Δ	As for BY4741, *ste24*Δ::*kanMX4*	Euroscarf (Winzeler *et al*., 1999)
BY4742 *ste24*Δ	As for BY4742, *ste24*Δ::*kanMX4*	Euroscarf (Winzeler *et al*., 1999)
BY4741 *chs4*Δ	As for BY4741, *chs4*Δ::*kanMX4*	Euroscarf (Winzeler *et al*., 1999)
BY4742 *chs4*Δ	As for BY4742, *chs4*Δ::*kanMX4*	Euroscarf (Winzeler *et al*., 1999)
BY4741 *rce1*Δ	As for BY4741, *rce1* Δ::*kanMX4*	Euroscarf (Winzeler *et al*., 1999)
BY4742 *sst2*Δ	As for BY4742, *sst2* Δ::*kanMX4*	Euroscarf (Winzeler *et al*., 1999)
CEN.PK2 -1D	*MAT*α *ura*3-52 *trp*1-289 *leu*2-3_112 *his3*Δ1 *MAL2*-8^{C} *SUC2*	Euroscarf (Entian *et al*., 1999)
CEN.EN13-3C *ste24*Δ	As for CEN.PK2, *YJR117w::HIS3*	Euroscarf (Entian *et al*., 1999)
Ste24TAP	*MAT*a *ade2 arg4 leu2*-3_112 *trp*1-289 *ura*3-52	CellZone AG (Heidelberg, Germany)
Chs3GFP	As for BY4741, *CHS3-GFP::HIS3*	Invitrogen (Huh *et al*., 2003)
Ste24GFP	As for BY4741, *STE24-GFP::HIS3*	Invitrogen (Huh *et al*., 2003)
Chs3GFP *ste24*Δ	As for BY4741, *CHS3-GFP::HIS3 ste24*Δ::*kanMX4*	This study
Chs3GFP *chs4*Δ	As for BY4741, *CHS3-GFP::HIS3 chs4*Δ::*kanMX4*	This study
*rce1*Δ *ste24*Δ	As for BY4741, *rce1*Δ::*kanMX4, ste24*Δ::*HIS3*	This study
AH109	*MAT*a *trp*1-901 *leu*2-3_112 *ura*3-52 *his*3-200 *gal4*Δ *gal80*Δ *LYS2*::*GAL1*$_{UAS}$-*GAL1*$_{TATA}$-*HIS3 GAL2*$_{UAS}$-*GAL2*$_{TATA}$-*ADE2 URA3*:: *MEL1*$_{UAS}$-*MEL1*$_{TATA}$-*lacZ MEL1*	Clontech (James *et al*., 1996)
BY4741 CHS4^{C693S}	As for BY4741, CHS4C693G chs4-terminator:*kanMX4*	This study

Table 5. Yeast strains used in this work

2.1.6 Plasmids

Plasmid	Description	Source/reference
pAG503	centromeric, G418 resistance	Laboratory collection
pAG503 *CHS3*	Chs3 under endogenous promoter control	This study
pAG503 *GAL-STE24*↑	Ste24 under Gal1 promoter control	This study
pRS415	centromeric, *Leu2*	Stillman D. J.
pRS415ste24	Ste24, endogenous promoter	This study
pRS415ste24^{E269G}	Ste24^{E269G}, endogenous promoter	This study
pRS415 *CHS3* Myc	Chs3, endogenous promoter, C-terminal 13Myc-tag	This study
pGREG576	centromeric, G418 resistance, *Ura3*, Gal promoter, GFP	Euroscarf (Jansen *et al*., 2005)
pGREG576 CHS4	Chs4, Gal promoter, N-terminal GFP	This study
pGREG576 CHS4^{C693S}	Chs4^{C693S}, Gal promoter, N-terminal GFP	This study
pJJH71	2µ,*PFK2* promoter, *Ura3*	(Raben *et al*., 1995)
pJJH71 ScSTE24	STE24, *PFK2* promoter	This study
pJJH71 ZmpSTE24	ZmpSTE24, *PFK2* promoter	This study
pJJH71 TcSTE24	TcSTE24, *PFK2* promoter	This study
pFA6a-GFP(S65T)-KanMX4	G418 for homologous recombination	Laboratory collection
pGADT7	Bait vector, Amp^r, *LEU2,*	Clontech
pGAD-C1	Bait vector, chs3 aa 1-165 (C1)	This study
pGAD-C3	Bait vector, chs3 aa 226-452 (C3)	This study
pGAD-C4	Bait vector, chs3 aa 476-1000 (C4)	This study
pGAD-C7	Bait vector, chs3 aa 1109-1165 (C7)	This study
pGAD-S2	Bait vector, ste24 aa 36–95 (S2)	This study
pGAD-S6	Bait vector, ste24 aa 221-304 (S6)	This study
pGAD-S8	Bait vector, ste24 aa 384-453 (S8)	This study
pGBRT7	Prey vector, Kan^r, *TRP1*,	Clontech
pGBR-C1	Prey vector, chs3 aa 1-165 (C1)	This study
pGBR-C3	Prey vector, chs3 aa 226-452 (C3)	This study
pGBR-C4	Prey vector, chs3 aa 476-1000 (C4)	This study
pGBR-C7	Prey vector, chs3 aa 1109-1165 (C7)	This study
pGBR-S2	Prey vector, ste24 aa 36–95 (S2)	This study
pGBR-S6	Prey vector, ste24 aa 221-304 (S6)	This study
pGBR-S8	Prey vector, ste24 aa 384-453 (S8)	This study
pAG503 *SM*ChsA	Chimeric Chs under Chs3 endogenous promoter control	This study
pAG503 *SM*ChsB	Chimeric Chs under Chs3 endogenous promoter control	This study
pAG503 *SM*ChsC	Chimeric Chs under Chs3 endogenous promoter control	This study

Table 6. Plasmids used in this work

2.1.7 Oligonucleotides

Primer	Sequence 5' →3'	Amplified sequence
Primer for amplifying different cDNAs		
chs3pro(*Kpn*I)-F	TACTCAGGTACCAAAACGCGTTATTGGCGCTGG	*chs3* and *chs3* promoter
chs3(*Sac*II)-R	TACTCACCGCGGTGCAACGAAGGAGTCACTTTC	*chs3* without stop codon
chs3(*EcoR*I)-R	TACTCAGAATTCAAAACGCGTTATTGGCGCTGG	*chs3*
13Myc(*Sac*II)-F	TACTCACCGCGGCCCGGGTTAATTAACGGT	13Myctag
13Myc(*Sac*I)-R	TACTCAGAGCTCTTCACTAGTGATTGATAAATT	13Myctag
GALpro(*Kpn*I)-F	TACTCAGGTACCAGATCTGTAAAGAGCCCCATT	GAL1 promoter
GALpro_ATG_Flag (*Sal*I)-R	TACTCAGTCGACCTTGTCGTCATCGTCTTT GTAGTCAGACATTTTGAGATCCGGGTT	GAL1 promoter
GFP(*Sac*II)-F	TACTCACCGCGGCGGATCCCCGGGTTAATTAA	*gfp*
GFP(*Sac*I)-R	TACTCAGAGCTCTATTCTGGGCCTCCATGTCGC	*gfp*
ste24-F	TACTCAGTCGACTTTGATCTTAAGACGATTCTC	*ste24*
ste24-R	TACTCACCGCGGTTAGTTTTTCTTCTTTTCACT	*ste24*
ste24-F(BamHI)	TACTCAGGATCCATGTTTGATCTTAAGACGATT	*ste24*
ste24_9His-R(SpeI)	TACTCAGCATGCCTAGTGGTGGTGGTGGTGGTGGT GGTGGTGGTTTTTCTTCTTTTCACT	*ste24*
Zmpste24(BamHI)-F	TACTCAGGATCCATGGGGATGTGGGCATCGCTG	*Zmpste24*
Zmpste24(SphI)-R	TACTCAGCATGCTCAGTGTTGCTTCATAGTTTT	*Zmpste24*
Tcste24(BamHI)-F	TACTCAGGATCCATGATCACCTTAAACGAGGTT	*Tc_ste*24
TcSTE24(SphI)-R	TACTCAGCATGCTTAGTCCTTCTTCTGCAGTGC	*Tc_ste*24
Primer for homologous recombination		
chs4^{C693S}-F	AGAAGGATAAACAAGGTAAAAAAAAAAAAGA CTCTGTAATTATGTAGGGCGCGCCAC TTCTAAA	*chs4*^{C693S}
chs4^{C693S}-R	GTGTAAACTGTTGCACCTATAAAGAATGAAAACAATC TAGCCGCATAGGCCACTAGTGGATCTG	*chs4*^{C693S}
Primer for site directed mutagenesis		
ste24^{E269G}-F	CGGCTGTTTTGGCCCATGGAATCGGTCACTGGC	*ste24*^{E269G}
ste24^{E269G}-R	GCCAGTGACCGATTCCATGGGCCAAAACAGCCG	*ste24*^{E269G}
Primer for yeast two-hybrid analyses		
chs3-C1 (*EcoR*I)-F	TAGTCAGAATTCATGACCGGCTTGAATGGAGAT	*chs3*-C1 (aa 1-165)
chs3-C1 (*BamH*I)-R	TACTCAGGATCCTGTATCATTCGTTTCCTTTCT	*chs3*-C1 (aa 1-165)
chs3-C3 (*EcoR*I)-F	TAGTCAGAATTCACCGTTTGTAGTAGTTCGAAA	*chs3*-C3 (aa 226-452)
chs3-C3 (*BamH*I)-R	TAGTCAGGATCCATCAGAGGCAATACAACCGAC	*chs3*-C3 (aa 226-452)
chs3-C4 (*EcoR*I)-F	TAGTCAGAATTCCGTTGGACTGTAGCTAGGAAA	*chs3*-C4 (aa 476-1000)
chs3-C4 (*BamH*I)-R	TAGTCAGGATCCCGTAGAATTAATCCATCTTCG	*chs3*-C4 (aa 476-1000)
chs3-C7 (*EcoR*I)-F	TAGTCAGAATTCAAATTTGATGACTTCTCATGG	*chs3*-C7 (aa 1109-1165)
chs3-C7 (*BamH*I)-R	TAGTCAGGATCCTGCAACGAAGGAGTCACTTTC	*chs3*-C7 (aa 1109-1165)
ste24-S2 (*EcoR*I)-F	TAGTCAGAATTCAGACAGTACCAGAAGCTATCT	*ste24*-S2 (aa 36–95)

ste24-S2 (*BamH*I)-R	TACTCAGGATCCTTTAGGGAAGAGGTCGTATTT	*ste24*-S2 (aa 36–95)
ste24-S6 (*EcoR*I)-F	TAGTCAGAATTCTTTAATAAGTTCACTCCATTG	*ste24*-S6 (aa 221-304)
ste24-S6 (*BamH*I)-R	TACTCAGGATCCTTTTTGCCAGTGACCGATTTC	*ste24*-S6 (aa 221-304)
ste24-S8 (*EcoR*I)-F	TAGTCAGAATTCAGTTTAATTTCCAGAACTCAT	*ste24*-S8 (aa 384-453)
ste24-S8 (*BamH*I)-R	TACTCAGGATCCGTTTTTCTTCTTTTCACTAAC	*ste24*-S8 (aa 384-453)
chs3(1428)-R	TACTCAGGATCCGAAGTAGCAGGCAATTATGAA	binds *CHS3* at bp 1428
chs3(2623)-R	TACTCAGGATCCAAGTCAAAGGTTGAAGCACGC	binds *CHS3* at bp 2623
chs3(3634)-R	TACTCAGGATCCAATTGCAGTTACCCAAGATTG	binds *CHS3* at bp 3634
Mschs2(1279)-F	TACTCAGGATCCATGAGAAATGCAGACCCTTGC	binds *MsCHS2* at bp 1279
Mschs2(1396)-F	TACTCAGGATCCTTATTGTGGCTCATATCTCAA	binds *MsCHS2* at bp 1396
Mschs2(2298)-F	TACTCAGGATCCCAGATGTTCGAGTATGCTATT	binds *MsCHS2* at bp 2298
Mschs2(2712)-R	TACTCACGGCCGGCACTGATAGACGATATACAG	binds *MsCHS2* at bp 2712
chs3C-t(4085)-F	TACTCACGGCCGTTGATTGGTACTATGGTACTG	binds *CHS3* bp at bp 4085

Table 7. Primer used in this work

2.1.8 Animals

Larvae of *M. sexta* (Lepidoptera, Sphingidae) were reared under long-day conditions (16 h of light) at 27°C using a synthetic diet modified according to Bell and Joachim (1974).

2.2 Molecular biological and genetic methods

2.2.1 Electrophoresis, isolation and quantification of DNA

Electrophoretic separation of DNA-fragments was carried out using 0.7% to 2.0% (w/v) agarose gels and TAE-buffer systems. DNA samples were supplemented with 1/5 volume of sample buffer (f.c. 40 mM Tris, 10 mM NaAc, 1 mM EDTA, pH 8) and separated at a constant voltage (5–7 V/cm electrode distance). A molecular mass marker (Smart Ladder, Eurogentec) migrated among the DNA-samples to evaluate the respective sizes. Following

electrophoresies the gels were incubated for 30 min in 1% (w/v) ethidium bromide solution (Sigma; f.c.100 µl/mg) and washed in H_2O_{deion}. Stained nucleic acids were visualized using a Versa Doc Imaging System (λ_{ex}=356 nm, 520LP filter, BioRad Laboratories) and the Quantity One v4.6 software (BioRad Laboratories). If required, DNA-fragments were purified using the "Qiaquick Gel Extraction Kit" (Qiagen, Hilden, Germany) according to manufacturer's specifications. Purification of DNA after enzymatic reactions (digestion, ligation or polymerase chain reaction, PCR) was carried out using the "QIAquick PCR Purification Kit" (Qiagen), while isolation of plasmid-DNA was done with the "QIAprep Miniprep Kit" (Qiagen) following the manufacturer's protocols. DNA concentration was determined by extinction measurements at 260 nm; an OD_{260} equates 50 ng/µl double strand DNA.

2.2.2 Plasmid construction

All DNA manipulations were performed in *E. coli* DH5α (Hanahan, 1983) according to Sambrook *et al*. (2000). Genes of interest were amplified from genomic *S. cerevisiae* DNA or from *M. sexta* cDNA using the PCR-Method (Saiki *et al*., 1988). Amplifications were performed using either "Taq DNA-Polymerase" (NEB), "Phusion® High-Fidelity DNA Polymerase" (NEB) or "Expand High Fidelity Taq-Polyermase" (Roche) according to the manufacturer's specifications. Site directed mutagenesis using the "QuickChange kit" (Stratagene) was also done according to the manufacturer's instructions. Plasmid inserts sequences were verified by nucleotide sequencing. Plasmid constructs and used oligonucleotides are listed in Table 7.

2.2.3 Transformation of yeast cells

To ensure optimal efficiency, yeast transformation was done by electroporation. *S. cerevisiae* cells were grown in YPD or minimal synthetic defined (SD) media until the early logarithmic phase was reached and washed twice in H_20_{deion}. Following a third wash in 1 M sorbitol, cells were transformed via electroporation (1 pulse, 1.5 kV, 25 µF, 200 Ω) with 1 µg plasmid DNA per

transformation. The procedure was performed in 50 µl 1 M sorbitol using a GenePulserXcell (BioRad). Subsequent to transformation, yeasts were incubated in YPDS-medium for 2 h at 30°C and 150 rpm, washed in H_20_{deion} and streaked onto selective media plates.

2.2.4 Generation of yeast strains

All strains used in this study are listed in Table 5. BY4741, CEN.PK and their isogenic knockout strains were obtained from EUROSCARF (Frankfurt, Germany). GFP-fusion protein strains were obtained from Invitrogen (Carlsbad, USA). Genomic point mutations were generated by homologous recombination using adequate primer and the vector pFA6a-GFP(S65T)-KanMX4 as a template (McElver and Weber, 1992). Following transformation of BY4741 cells and selection on appropriate growth media, the point mutation was verified by sequencing the PCR-generated fragment. Double mutants were constructed by mating of single mutant strains. Resulting diploid strains were sporulated, and segregants carrying double mutations were grown on selective media plates. The double mutations and mating types were confirmed by direct PCR (Huxley *et al*., 1990).

2.2.5 Total-RNA isolation and reverse transcription

To minimize RNA degradation, glassware was sterilized over night at 200°C or treated with "RNase away" (Invitrogen). Total-RNA was isolated from one *M. sexta* midgut fifth instar larva on a cooling plate. After removing Malpighian tubules, peritrophic matrix and the gut content, the midgut tissue was washed and homogenized for 1 min with an Ultraturrax (T25, Janke & Kunkel) at 25,000 rpm. Subsequently total-RNA-isolation was done with the “RNA-easy Mini Kit” (Qiagen) according to manufacturer’s instructions. Isolated RNA was eluted in DEPC-H_2O. The subsequent cDNA-first strand synthesis was performed using the “First Strand cDNA Synthesis Kit for RT-PCR (AMV)" (Roche) according to manufacturer's protocol. The cDNA was either stored at –20°C or used as PCR template immediately.

2.2.6 Yeast two hybrid analysis

To analyze putative protein interactions, yeast two hybrid analysis was performed with the "Matchmaker two-hybrid system" according to the manufacturer's protocol and the "Yeast Protocol Handbook" (Clontech). To identify interacting proteins and hydrophilic domains mediated binding, the complete ORF of soluble proteins or hydrophilic domains from membrane proteins were amplified with specific primers (Table 7) and individually ligated into the yeast expression vectors pGADT7 and pGBKT7 (Table 6). After testing all constructs for endogenous activation of reporter gene activity as a negative control, yeast cells of the strain AH109 were co-transformed with prey and bait plasmids by electroporation (Table 6).

Cells were incubated overnight in liquid SD $^{-Leu\ -Trp}$ medium at 30°C and diluted with water to a final concentration of 1 x 10^7 cells/ml. Five µl of each suspension and three subsequent 10-fold serial dilutions were individually spotted onto SD$^{-Leu\ -Trp}$, SD$^{-Leu\ -Trp\ -His}$ and SD$^{-Leu\ -Trp\ -His\ -Ade\ +X\alpha Gal}$ plates for selection. Cells were incubated at 30°C for 2 days.

2.2.7 Halo assays

The production of mature **a**-factor from various MAT**a** strains was monitored by pheromone diffusion (halo) assay, modified after Trueblood *et al*. (2000). MAT**a** wild-type cells were dropped at high concentrations onto agar plates with low concentrated MATα cells. Mating deficiency is indicated by growth inhibition of the MATα cells leading to the formation of a halo surrounding the MAT**a** drops. To prepare a homogenous cell suspension, approximately 5 × 10^6 cells of the MATα sst2 strain were mixed with 3 ml YPD media (42°C) containing 0.7 % (w/v) agarose (molecular grade, Bioline) and 0.04% Triton X-100 and streaked onto solid rich medium (YPD) plates containing 0.04% Triton X-100.

2.2.8 Calcoflour white serial drop dilution assay

Calcoflour white (CFW) is a fluorescent dye that binds primarily to chitin (Roncero and Duran, 1985). Since this compound is toxic to yeast cells, mutants with reduced chitin levels exhibit a CFW resistance phenotype. CFW growth tests are an easy and fast method to evaluate chitin levels. *In vivo,* the vast majority of chitin deposited in the yeast cell wall is synthesized by Chs3 (Shaw *et al*., 1991). Correspondingly, cells without a functional chitin synthase 3 produce significantly less chitin and are CFW resistant. Therefore, changes in CFW resistance can be correlated with changes in Chs3 activity. Each 5 µl of a yeast suspension were spotted at different concentration (10^3-10^7cells/ml) onto solid rich medium (YPD or YPG) plates containing 50 µg/ml CFW. Following incubation at 30°C for 3 days, colony growth was documented using a Versa Doc Imaging System (BioRad) and the Quantity One software (version 4.6; BioRad Laboratories).

CFW tends to precipitate in media plates over time and loses its toxicity. Therefore only freshly prepared CFW media plates were used to analyze cell resistance and appropriated control stains were included.

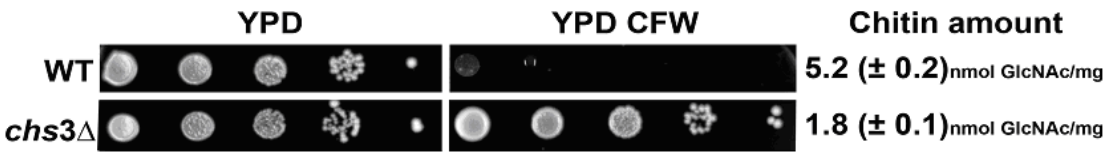


Figure 8. CFW phenotypes correlate with chitin amounts - Wild-type and *chs3*Δ cells were grown overnight in liquid full medium and diluted with water to a final concentration of 1 x 10^7 cells/ml. Five µl of each suspension and four subsequent 10-fold serial dilutions were spotted onto YPD media plates with or without 50 µg/ml CFW. Cells were incubated at 30°C for 3 days. Chitin amounts were additionally quantified by the Morgan-Elson method. Data represent averages (± S.E.) obtained from six independent experiments for each yeast strain.

2.2.9 CFW fluorescence assay

To evaluate relative chitin amounts in the yeast cell wall, a CFW-fluorescence assay based on the specific binding of CFW was performed modified according to Lam *et al*. (2006). Each 5 µl of a concentrated yeast suspension (10^9 cells/ml) were spotted onto solid rich medium (YPD or YPG) plates containing

50 µg/ml CFW. Following incubation at 30°C for 3 days, the fluorescence was quantified densitometrically using a Versa Doc Imaging System (λ_{ex}=356 nm, 520LP filter, BioRad Laboratories) and the Quantity One v4.6 software (BioRad Laboratories). The optical densities from constant areas within the spots were averaged over 15 to 40 independent experiments and corrected for the local background. The mean optical density averaged over 40 spots of wild-type cells was set to 100%, while the mean optical density averaged over 40 spots of *chs*3Δ cells was set to 0%. Relative chitin amounts (RCA) from various mutants were calculated according to RCA = $(OD_x\text{-}OD_{chs3\Delta})/(OD_{WT}\text{-}OD_{chs3\Delta})$, with x being the mean optical density averaged over 20 spots of the respective yeast mutant.

2.3 Protein biochemical methods

2.3.1 Determination of protein concentration

Concentrations of proteins in solution were determined utilizing the “Amido Black” method (Wieczorek *et al*., 1990). This method is based on a chemical reaction between the acid Amido Black with alkaline amino groups of proteins. In contrast to other methods, the Amido Black method is relative resistant to detergents and many buffer systems used in biochemistry. Protein solutions of interest along with Bovine serum albumine (BSA) solutions as a standard (2, 4, 6 µg) were incubated with Amido Black solution (26 mg/100 ml in 10% (v/v) acetic acid, 90% methanol) for five minutes. Following centrifugation (16000 x g, 4 min) at room temperature, samples were washed twice with washing solution (10% (v/v) acetic acid, 90% methanol). Resulting pellets were solubilized in 100 mM NaOH and extinction was measured photometrically at 615 nm. Protein concentrations were calculated by comparison with known BSA concentrations.

2.3.2 SDS-PAGE and protein staining

SDS-PAGE was performed to determine the apparent molecular masses of unknown proteins using a discontinuous buffer system (Laemmli, 1970). According to their amino acid composition, proteins migrate individually in SDS-gels with their migration speed being directly proportional to the logarithm of their molecular weights. Gel electrophoresis was done using the "Mini-PROTEAN II system" (BioRad, Munich, Germany). Native-PAGE was performed with discontinuous polyacrylamide gels (ReadyGel 10–20% Tris–HCl, pH 8.3) following the manufacturer's recommendations (BioRad). If necessary, separated proteins were transferred onto nitrocellulose membranes (Western Blot, 2.3.3) or stained with Coomassie Blue (Schweikl *et al*., 1989). SDS-Gels were documented using a Versa Doc Imaging System (BioRad) and the Quantity One software (version 4.6; BioRad).

2.3.3 Western blot and immunostaining

To identify specific proteins via immunostaining, the proteins were separated by SDS-PAGE or Native-PAGE and transferred onto nitrocellulose membrane (Trans Blot®, 0.45 µm, BioRad) using the semi dry method (Towbin *et al*., 1979). Protein transfer was performed by a Trans Blot-SD System (BioRad) in a tripartite buffer system according to Kyhse-Anderson (1984). The buffer system was modified by supplementation of 20% (v/v) methanol for enhanced transfer of membrane proteins. Following 1 h of protein transfer (1 mA/cm2 membrane area), the membranes were reversibly stained with a 0.002% (w/v) Ponceau-S solution (Sigma) and documented.

Specific detection of transferred proteins was performed via immunostaining. Subsequent to 1 h of blocking in an adequate buffer (5% (w/v) milk powder in TBS (20 mM Tris/HCl, 500 mM NaCl, pH 7.5)), membranes were incubated for one hour at room temperature in primary antibody solution (2.5% (w/v) milk powder in TBS including the primary antibody at a concentration is listed in Table 2). Unspecific and unbound antibodies were removed by threefold washing (5 min each) in TBS-Tween (0.05% (v/v) Tween20 in TBS). Subsequently the membrane was incubated for 1 hour in secondary antibody

solution (specific concentration is listed in Table 2) and washed again threefold in TBS-Tween. Following equilibration of the membrane in APP-buffer (100 mM NaCl, 50 mM $MgCl_2$, 100 mM Tris/HCl, pH 9.5), the membranes were stained with NBT/BCIP solution (45 µl NBT, 35 µl BCIP in APP-buffer; NBT: 75 mg/ml NBT in 70% (v/v) DMF; BCIP: 50 mg/ml BCIP in 100% DMF). The enzymatic reaction (alkaline phosphatase) was stopped by washing in H_2O_{deion} after a few minutes depending on the intensity of the staining.

2.3.4 Yeast protein extraction

Cells were grown in complete (YPD, YPG) or synthetic defined (SD) media until the early logarithmic phase was reached. Subsequently, cells were harvested by centrifugation (3 min at 3,000 x g and 4°C) and the resulting pellet was resuspended in 50 mM Tris-HCl buffer (pH 7) containing 20 mM NaCl and one tablet “Complete Protease Inhibitor Cocktail” (Roche) in 50 ml buffer. After adding the same volume of glass beads, cells were vortexed for 5 min at 4°C. Following another centrifugation step (3 min at 3000 x g and 4°C) the resulting pellet was supplemented with Laemmli buffer and boiled for 1 min (Laemmli, 1970).

2.3.5 Purification of *M. sexta* midgut chitin synthase

Chitin synthase of the *M. sexta* midgut was purified according to Maue *et al*. (2009). In brief isolated midguts of thirty *M. sexta* fifth instar larvae were dissected on a cooling plate. Tissues were washed and homogenized.

In the first purification step, the membrane fraction was separated from soluble proteins. *M. sexta* midgut homogenates were differentially centrifuged in a fixed-angle rotor (10 min at 600 x g, 30 min at 10,000 x g and 1 h at 100,000 x g). To solubilize the chitin synthase, the membrane vesicles of the 10,000 x g pellets were resuspended in solubilization buffer (50 mM MOPS, 10 mM $MgCl_2$, 30 mM KCl, pH 6.5, 0.5% (v/v) Triton X-100) and incubated for 20 min at 30°C. To enrich the chitin synthase from the solubilized midgut fraction, the respective sample was fractionated by discontinuous sucrose density gradient

centrifugation. The proteins of the 10% (w/v) sucrose fraction were diluted 1:10 in a low salt buffer (20 mM Tris–HCl, 50 mM NaCl and 0.025% (v/v) Triton X-100, pH 8.1) and subjected to anion exchange chromatography (MonoQ 5/5 HR, GE Amersham). The chitin synthase containing fractions eluted at 230-360 mM NaCl and were pooled and concentrated. The chitin synthase containing concentrate was injected into a Superdex 200 HR 10/300 GL gel chromatography column (GE Amersham). To prevent proteolytic degradation, the eluted chitin synthase was supplemented with 5 mM Pefabloc SC (Serva).

2.3.6 Measurement of chitin synthesis

Chitin synthesis was measured according to Zimoch *et al*. (2005). Standard assay was performed in 100 ml of CHS-buffer. The reaction was initiated by the addition of 30 nCi UDP-N-acetyl-D-[U-^{14}C]-glucosamine (specific activity: 285 mCi/mmol, GE Amersham). Incubation was carried out for variable time periods in 1.5 ml Eppendorf polypropylene tubes at 30°C. The reaction was stopped by adding 1 ml 10% (w/v) trichloroacetic acid. Insoluble material was collected by glassfiber filters (PALL; Typ A/E, 0.3 µm). Filters were subsequently washed with 10 ml 98% ethanol and dried using an infrared lamp. Dried filters were transferred into scintillation tubes and 5 ml Ecolume scintillation liquid (ICN) was added. Radioactivity (counts per minute, cpm) was measured in a liquid scintillation counter (Beckman LS6500). Each experiment was repeated at least three times with each single value being determined in triplicates. To ensure that the synthesized macro molecules were mainly chitin, dried filters were incubated for 2 h at 100°C in 1.5 M KOH (Londershausen *et al*., 1988).

2.3.7 Measurement of chitin content

The chitin content of different yeast cell strains was determined by the Morgan-Elson method as described in Bulik *et al*. (2003) with some minor modifications. Cultures for chitin determination were made from those initially grown to stationary phase in liquid YPD medium and then diluted 1:100 in fresh medium and incubated at 30°C with shaking for 18 to 22 h. After 1 ml of culture was

centrifuged for 5 min at 3,000 rpm, washed and all residual liquid was removed from the pellet, the wet weight of the cells was determined. The cell pellets were suspended in 0.5 ml of 6% (w/v) KOH and heated at 80°C for 90 min. KOH treated cell were incubated for 48 h with 5 μl (20 mg/μl) *Streptomyces griseus* chitinase (Sigma-Aldrich). Colorimetric determination of GlcNAc was performed in microtiter plates, of which each slot was loaded with 150 µl of the samples treated with Ehrlich's reagent, and absorbance at 585 nm was recorded. Standard curves were prepared from stocks of 0.2 to 2.0 mM GlcNAc.

2.4 Microscopy

In general, cells were grown in YPD or minimal synthetic defined (SD) media until the early logarithmic phase was reached. If gene expression was controlled by a *GAL1* promoter, cells were grown in glucose-free medium containing raffinose as a carbon source and analyzed in early logarithmic phase three hours after induction of gene expression by adding 2% galactose (w/v) to the medium. For CFW staining, yeast cells were incubated in 0.02 % CFW solution (w/v) for 30 min at room temperature and washed three times with deionized water. Microscopy was performed with a 100x oil-immersion objective (NA 1.36) and an IX70 fluorescence microscope (Olympus, Hamburg, Germany). Fluorescence was excited with a U-RFL-burner (Olympus) and appropriate filter cubes were used to set excitation and emission wavelengths. Images were captured with a CoolSNAP HQ2 digital camera (Roper Scientific, Tucson, USA) using MetaMorph 6.2 software (Molecular Devices, Toronto, Canada). Z-stack series of 12 optical layers were taken for each analyzed cell. Protein distribution was analyzed from Z-stack series comprising between 150 and 250 single cells.

2.5 Other methods

Alignment of amino acid sequences was performed with ClustalW (Higgins, 1994). Prediction of transmembrane helices was performed with TMHMM

Server v. 2.0 and ProtScale (ExPASy Proteomics Server). Identification of known phosphorylation sites were performed PhosphoGRID and PhosphoPep Database. Prediction of phosphorylation sites and palmitoylation sites were performed with NetPhosYeast 1.0 Server and with CSS-Palm2.0, respectivly.

3 Results

3.1 The *M. sexta* chitin synthase

3.1.1 Introduction

Several studies have suggested that insect and fungal chitin synthases are produced as zymogens requiring proteolytic cleavage for activation (Merzendorfer, 2006). Duran *et al*. (1975) first described the zymogenic properties of a chitin synthase. However, due to the lack of suitable purification protocols for chitin synthases, the zymogenic character of these enzymes was demonstrated only in cell extracts or enriched membrane factions. Therefore it was not clear, whether the chitin synthase itself is a zymogene or if zymogenic activation is an indirect effect caused by an upstream regulatory protein. Zimoch (2007) established a protocol to purify active chitin synthase 2 protein complexes from the midgut of *M. sexta*. The availability of this protocol finally allows analyzing the susceptibility of a purified chitin synthase to zymogenic activation and its oligmeric properties.

3.1.2 The *M. sexta* chitin synthase 2 (MsChs2) complex

Purified chitin synthase turned out to exist as an oligomeric complex of about 520 kDa. Under denaturing conditions, the chitinsynthase complex dissolves into 5 proteins with the apparent molecular masses 180, 123, 79, 67 and 59 kDa, respectively. Interestingly, immunostainings utilizing an antibody to the catalytic domain of MsChs2 detected only the uncleaved 180 kD protein, a intermediate 123 kDa fragment and the 67 kDa catalytic domain, suggesting that the MsChs2 complex is cleaved twice (Maue *et al*., 2009). To provide additional evidence for its oligomeric properties, MsChs2 was isolated according to Maue *et al*. (2009) (2.3.5.) and subsequently analyzed by Native-PAGE. The Native-PAGE (Figure 9) of the purified protein showed only one high molecular weight band around 520 kDa. This protein was also immunoreactive with the antibody to the catalytic domain of MsChs2, confirming pervious observations suggesting that a trimeric complex was

purified. Subsequently, the MsChs2 complex was subjected to electron microscopy. As shown in Figure 10, particles of about 10 nm in diameter were visualized after a negative stain with uranylacetate. This size is in line with a complex of around 500 kDa. Some trimeric complexes form super complexes, as highlighted in Figure 10. These super complexes are composed of dimers of trimers (small picture left) or trimers of trimers (small picture button right). Unfortunately, the preparation was not homogeneous enough to perform single particle analsys, which might be due to the association of the enzyme with chitin fibrils, since the complex can be stained with CFW (Maue *et al*., 2009).

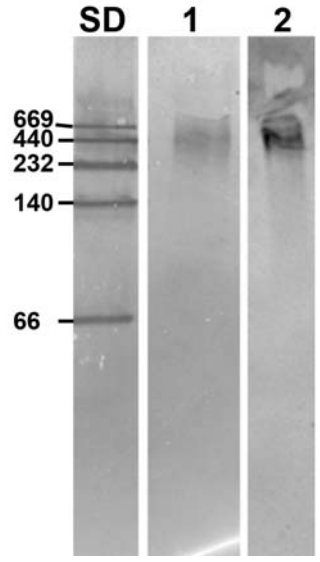


Figure 9. Purified MsChs2 complex – Native-PAGE; lane 1: Coomassie Blue stain; lane 2: immunostaining with anti-Chs-antibody after western blotting onto a nitrocellulose membrane; SD: Native high molecular weight standard.

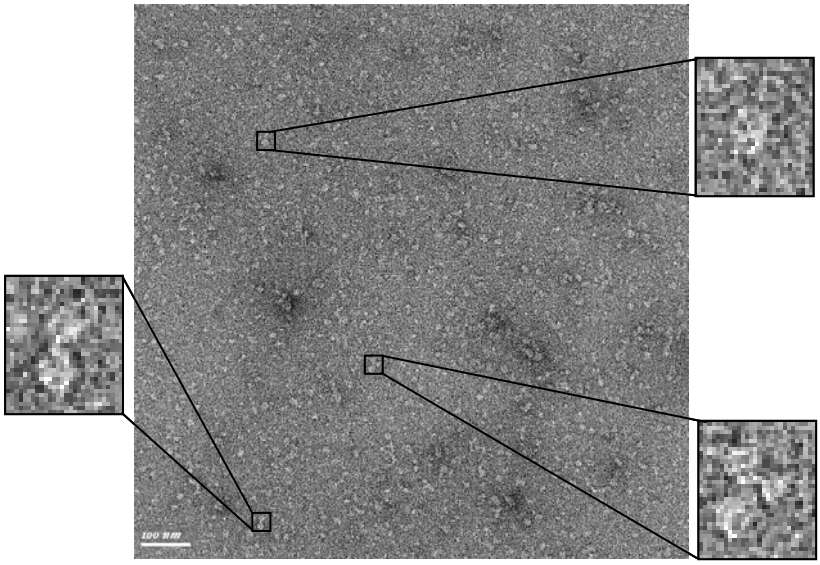


Figure 10. Transmission electron microscopy (TEM) of negativly stained MsChs2 complex – Micrograph was taken by Anke C. Terwisscha van Scheltinga at the Max Planck Institute of Biophysics in Frankfurt am Main, Germany. Small picture left: dimer of trimer, right top: single trimeric complex; right bottom: trimer of trimer.

3.1.3 Zymogenic properties of purified MsChs2

As reported previously, the activity of insect and yeast chitin synthases can be increased by adding proteases (Merzendorfer, 2006). However, this effect has never been shown on the level of the purified protein complex. To investigate, if this augmenting effect on chitin synthesis is also apparent after treatment of the purified enzyme, of the enzyme activity of purified MsChs2 was measured by incorporation of *N*-Acetyl-D-[U-^{14}C]-glucosamine following the addition of different proteases or inhibitors (see 1.3.6.).

The results in Figure 11 show the chitin synthase activity measurements. The mean value of synthesized chitin between 5 and 6 hours for MsChs2 without protease was 1450 cpm. If chymotrypsin (50 µg/ml) was added to the assay, the amount of incorporated *N*-Acetyl-D-[U-^{14}C]-glucosamine increased more than three fold; the addition of chymotrypsin and chymotrypsin inhibitor simultaneously decreased the synthesis rate (1.5 fold higher than without protease). By adding trypsin (50 µg/ml) to the chitin assay, the amount of incorporated *N*-Acetyl-D-[U-^{14}C]-glucosamine increased more than five fold compared to the chitin assay without protease, while additional trypsin inhibitor decreased the amount (1.7 fold higher than without protease). The results in

Figure 11 clearly show that the solubilized enzyme complex is readily activatable either by chymotrypsin or by trypsin. With respect to trypsin, these results were unexpected since Zimoch *et al*. (2005) have previously shown that trypsin stimulates chitin synthesis in crude midgut extracts but not in membrane fractions. Maybe restoration of trypsin activation is due to structural changes of the complex after solubilization or due to the loss of a regulatory subunit, as it is also observed for Chs3.

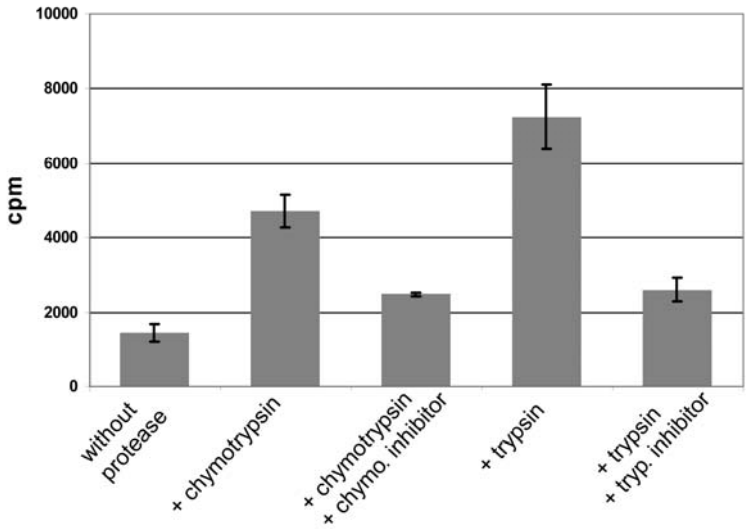


Figure 11. Activities of purified MsChs2 with and without addition of protease - Chitin synthesis of purified MsChs2 in the presence of proteases. Chitin synthesizing activity of purified MsChs2 was measured by incorporation of *N*-Acetyl-D-[U-^{14}C]-glucosamine. 0.2 μg of MsChs2 was incubated at 30°C for five and six hours, respectively. Graph displays the increase of incorporated *N*-Acetyl-D-[U-^{14}C]-glucosamine between five and six hours of incubation. Data represent averages (± S.E.) obtained from three independent experiments.

3.2 Chimeric chitin synthase

3.2.1 A yeast triple deletion mutant defective in all *chs* genes is viable in osmotically stabilized media

To investigate the *M. sexta* chitin synthase in a heterologous expression system, the complete cDNA of encoding this enzyme was cloned and expressed in *E. coli*, yeast cells, insect cells and Cos-cells, respectively (Zimoch, Gerdemann and Finsterbusch, personal communications). A

functional active chitin synthase, however, could not be expressed in any of these systems. Nevertheless, in order to analyze some of the catalytic properties of MsChs2, chimeric chitin synthases carrying the catalytic domain of MsChs2 were constructed and heterologously expressed in *S. cerevisiae*. To evaluate putative chitin synthesis, a triple deletion mutant for all three *chs* (*chs1*Δ, *chs2*Δ, *chs3*Δ) was used as expression system limiting chitin synthesis background. As previously reported, *S. cerevisiae* is viable in absence of chitin under osmotically stabilizing conditions (YPDS media) and at reduced temperature (26 °C; Schmidt, 2004). As seen in Figure 12, triple *chs*Δ mutant cells formed microcolonies indicating that the daughter cells fail to separate from their mothers (Schmidt, 2004). The cells grew very slowly with a generation time of about 7-8 h. The triple deletion mutants could not be transformed by chemical transformation protocols, probably due to cell wall instability. Therefore the triple deletion mutants were transformed by electroporation (2.2.3).

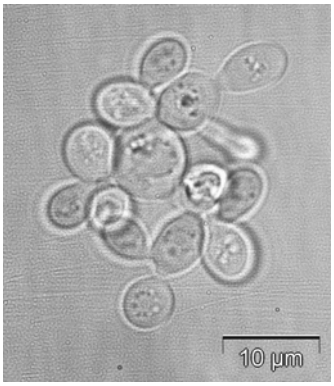


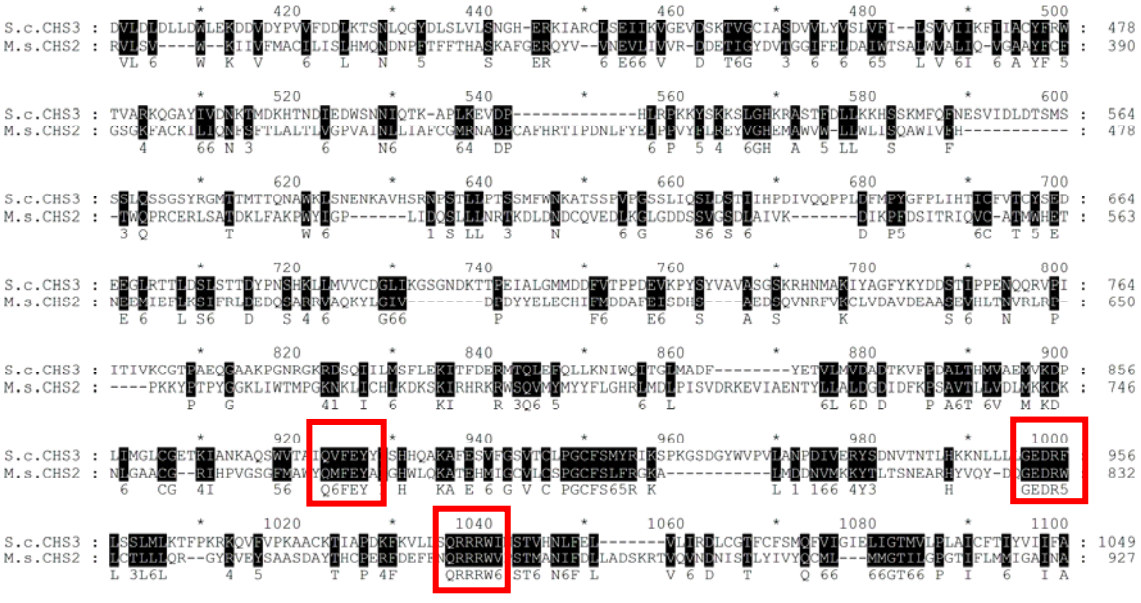
Figure 12. Micrographs of triple *chs*Δ mutants - Cells were grown in YPDS media at 26 °C. Triple chitin synthase deletion mutants are growth deficient. Mutant cells grow in microcolonies.

3.2.2 Construction of chimeric chitin synthases

The chitin synthase 3 of *S. cerevisiae* is one of the best described chitin synthases and its activity can easily be examined by CFW resistance tests (2.2.8). The two proteins Chs3 and MsChs2 differ considerably at their C- and N-termini, whereas the characteristic nucleotide, sugar and product binding motifs in the respective catalytic domains are conserved (Figure 13B). Therefore, 3 different yeast expression vectors were constructed carrying the ORF of Chs3 with the catalytic domain being exchanged by the catalytic

domain of MsChs2 (Figure 13A). The three expression vectors differ in the size of the inserted MsChs2 domain: the first chimeric chitin synthase (SMChsA) carries the complete predicted cytoplasmic B domain of MsChs2 including the catalytic domain (426-904 aa), whereas the second contains amino acids 465-904 of MsChs2 (SMChsB). In SMChsC only the amino acids belonging to the catalytic core (776-904 aa) were exchanged (Figure 13B). The chimeric chitin synthase expression vectors were constructed by sequential cloning of gene and cDNA fragments encoding the N-terminus of Chs3 including 350 bp of upstream sequence (endogenous promoter), different sizes of the cytoplasmic B domain of MsChs2 and finally the C-terminus of Chs3 into the single copy yeast vector pAG503. Triple deletion mutants in which all three chitin synthases were deleted (Schmidt, 2004; Figure 12) were transformed with the chimeric *CHS* expression vectors. As a control, triple deletion mutants were transformed with a vector carrying the wild-type Chs3 ORF under the control of its endogenous promoter. Successful transformation was controlled by amplifying the catalytic domains of MsChs2 utilizing PCR (Figure 13 C).

A



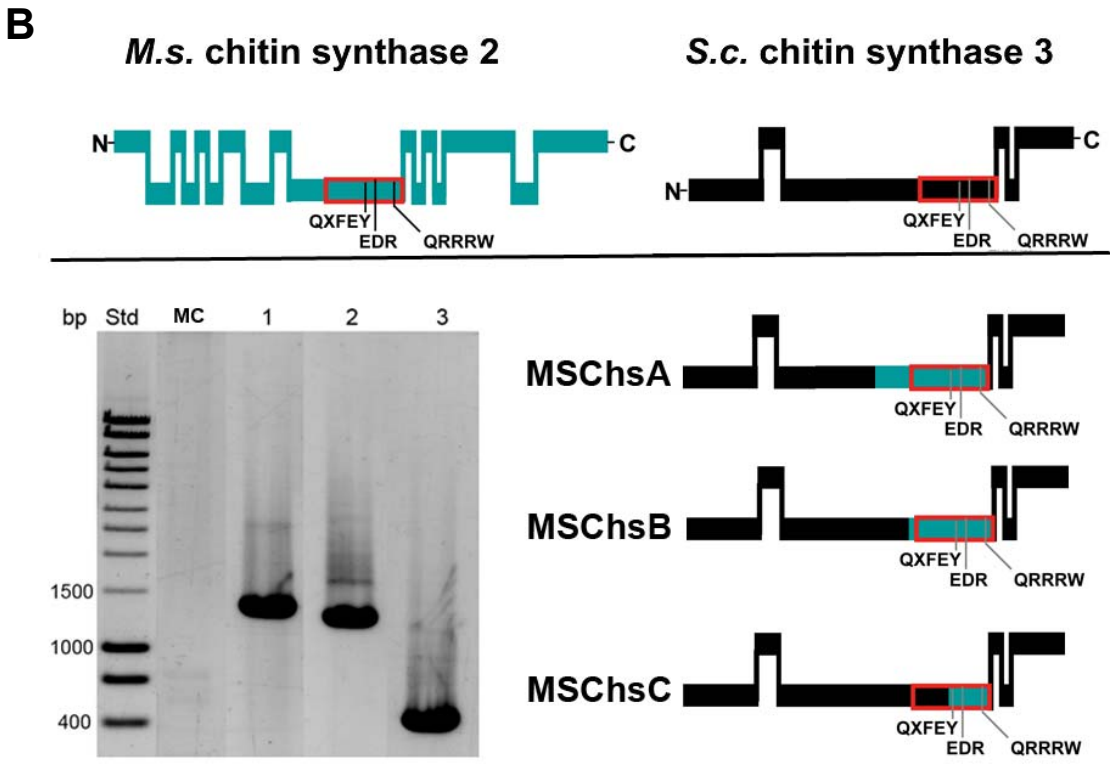


Figure 13. Domain shuffling – (A) Amino acid sequence alignment of the catalytic domains of chitin synthase 3 from *S. cerevisiae* and chitin synthase 2 from *M. sexta*. Red boxes indicate the conserved motifs. **(B)** Domain architectures of Chs3, MsChs2 and chimeric chitin synthases. Horizontal bars at the top represent extracellular domains, horizontal bars at the bottom intracellular domains, and vertical bars transmembrane helices. Direct PCR controlling the successful transformation with pAG503 SMChsA, pAG503 SMChsB, pAG503 SMChsC, respectively. Std, molecular mass marker (Smart Ladder, Eurogentec); MC, mock control using yeast cells transformed with the pAG503 chs3 vector; 1, *MsCHS2* catalytic domain of SMChsA; 2, *MsCHS2* catalytic domain of SMChsB; 3, *MsCHS2* catalytic domain of SMChsC. Red boxes indicate catalytic domain including the conserved motifs.

3.2.3 Chimeric chitin synthases do not restore CFW sensitivity in triple *chs* deletion strains

To test whether the chimeric proteins display chitin synthase activity, triple *chs* deletion mutants expressing chimeric Chs versions were analyzed for their sensitivity to CFW. As a control, triple *chs* deletion mutants carrying an empty expression vector or a vector carrying wild-type *CHS3* were also tested for CFW sensitivity. As expected, triple chs deletion mutant carrying an empty vector were resistant towards CFW while triple *chs* deletion mutants expressing *CHS3* exhibited significant sensitivity (Figure 14). Analysis of mutant cells carrying any of the chimeric chitin synthase vectors demonstrated that the

respective cells had the same resistance towards CFW as the empty vector control (Figure 14). None of the mutants expressing the chimeric proteins exhibited CFW sensitivity. Notably, even the chimeric chitin synthase *SM*ChsC, where only the core 118 aa of the catalytic domain of Chs3 were exchanged by the 128 aa of the catalytic domain of MsChs2, could not restore the CFW phenotype, although both sequences carry the chitin synthase consensus sequences and share 35% identical amino acids, which is higher than the identity between Chs3 and Chs1 or Chs2 (27% and 30%, respectively).

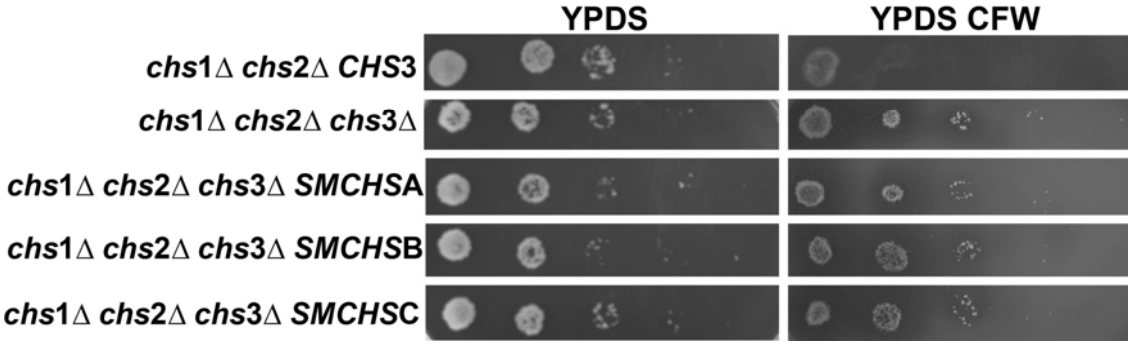


Figure 14. CFW resistance phenotypes of different yeast strains – *chs1*Δ *chs2*Δ *chs3*Δ, *chs1*Δ *chs2*Δ *CHS3*, *chs1*Δ *chs2*Δ *SMCHSA*, *chs1*Δ *chs2*Δ *chs3*Δ *SMCHSB*, *chs1*Δ *chs2*Δ *chs3*Δ *SMCHSC* cells were grown in liquid YPDS medium and diluted with water to a final concentration of 1 x 10^7 cells/ml. Five µl of each suspension and three subsequent 10-fold serial dilutions were spotted onto YPDS or YPDS plates with or without 50 µg/ml CFW. Cells were incubated at 26°C for 5 days.

3.3 Yeast chitin synthase 3

Several studies have suggested that insect and fungal chitin synthases including Chs3 are produced as zymogens requiring proteolytic cleavage for activation (Merzendorfer, 2006). However, in yeast up to now no protease has been identified which cleaves the zymogenic form of or stimulates chitin synthesis. Therefore, different databases were screened (BioGRID, BOND, BioPIXIE, DIP and Yeast RC Two hybrid) for proteases of any kind that could interact with yeast Chs3. Initially, 95 proteins that potentially interact with Chs3 were identified, but only one evident protease was detected, Ste24. It is a membrane-integral metalloprotease of the ER, which was identified to interact

with Chs3 in a large-scale split ubiquitin screen (Miller *et al*., 2005). More recently, two additional proteases were identified in a large-scale screen (Table 8; Tarassov *et al*., 2008).

Gene	Description	Experiment Type	Reference
APS1	subunit of the clathrin-associated adaptor complex AP-1	Affinity Capture-Western	(Copic *et al*., 2007)
ARV1	intermediate ER localization	PCA	(Miller *et al*., 2005)
BCH1	Member of the ChAPs family of proteins	Affinity Capture-Western	(Trautwein *et al*., 2006)
BCH2	Member of the ChAPs family of proteins	Affinity Capture-Western	(Trautwein *et al*., 2006)
BUD7	Member of the ChAPs family of proteins	Affinity Capture-Western	(Trautwein *et al*., 2006)
CHS3	Chitin synthase III	Two-hybrid	(DeMarini *et al*., 1997)
CHS5	Component of the exomer complex	Affinity Capture-Western	(Sanchatjate and Schekman, 2006)
CHS6	Member of the ChAPs family of proteins	Affinity Capture-Western	(Trautwein *et al*., 2006)
CHS7	Protein of unknown function	PCA	(Tarassov *et al*., 2008)
DNF1	Aminophospholipid translocase	PCA	(Tarassov *et al*., 2008)
DRS2	Aminophospholipid translocase	PCA	(Tarassov *et al*., 2008)
DTR1	Putative dityrosine transporter	PCA	(Tarassov *et al*., 2008)
ENT3	N-terminal epsin-like domain	Affinity Capture-MS	(Copic *et al*., 2007)
ENT5	N-terminal epsin-like domain	Affinity Capture-MS	(Copic *et al*., 2007)
EPT1	sn-1	PCA	(Miller *et al*., 2005)
FLC1	Putative FAD transporter	PCA	(Tarassov *et al*., 2008)
FLC2	Putative FAD transporter	PCA	(Tarassov *et al*., 2008)
KEX1	Protease	PCA	(Tarassov *et al*., 2008)
KEX2	Subtilisin-like protease	PCA	(Tarassov *et al*., 2008)
NMD2	involved in the nonsense-mediated mRNA decay	Affinity Capture-MS	(Krogan *et al*., 2006)
PFA4	Palmitoyltransferase with autoacylation activiy	Affinity Capture-Westen	(Lam *et al*., 2006)
RET2	Delta subunit of the coatomer complex	PCA	(Tarassov *et al*., 2008)
CHS4	Activator of Chs3	Two-hybrid	(DeMarini *et al*., 1997)
STE24	zinc metalloprotease	PCA	(Miller *et al*., 2005)
UBI4	Ubiquitin	Affinity Capture-MS	Peng *et al*. (2003)

Table 8. Proteins that physical interact with Chs3 – identivied using BioGRID$^{3.0}$ database

3.3.1 Yeast two hybrid interactions of Chs3, Chs4 and Ste24

To confirm the interaction between Ste24 and Chs3 with an independent method and particulrly map the interacting regions, a yeast two hybrid analysis testing different soluble domains of Chs3 and Ste24 was performed. Tam *et al*. (2001) investigated the topology of the multispanning membrane protein Ste24. The published topology model was used to identity the soluble domains of Ste24 (Figure 16).

Since no structural data was published on Chs3, computer-assisted programs were used to identify putitative soluble domains. The TMHMM topology model of Chs3 proposed in Figure 16 includes the results from the yeast two hybrid analysis. In addition, the soluble catalytic domain including the QRRRW motif was placed at the cytosolic site, since the catalytic domain of chitin synthases is reported to be cytosolic (Merzendorfer, 2006). As a positive control, Chs4 was included in the yeast two hybrid analyses, a known activator of Chs3, which was reported previously to interact with Chs3 in two independent studies (DeMarini *et al*., 1997; Ono *et al*., 2000).

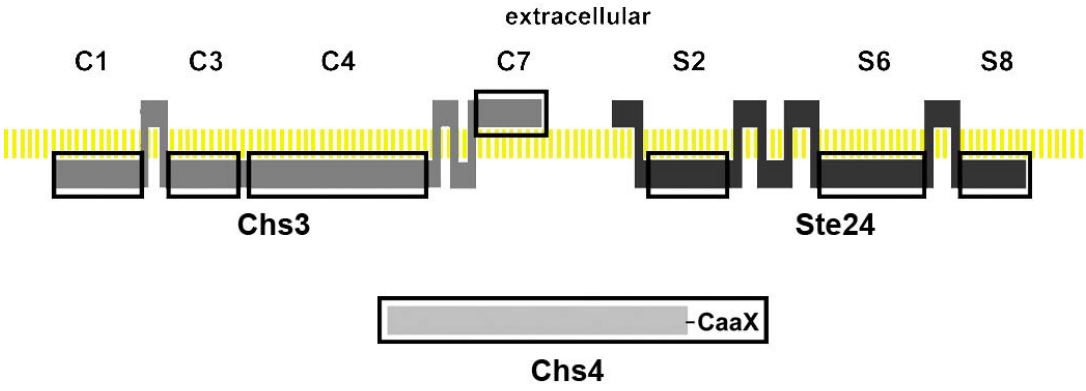


Figure 16. Putative domain architectures of Chs3, Chs4 and Ste24 - The yellow horizontal broken line represents the cell membrane. Horizontal bars at the top represent extracellular domain, horizontal bars at the bottom intracellular domains, and vertical bars transmembrane helices. Soluble domains tested in the yeast two hybrid analysis are marked with black squares.

The cDNA sequences encoding four soluble Chs3 domains, three soluble Ste24 domains and the complete coding sequence of Chs4 was amplified (Figure 16) and each of them was ligated into pGADT7-AD and pGBKT7-BD of the Matchmaker yeast two-hybrid system, in order to allow switching of bait and

prey inserts. After negatively testing all constructs for endogenous activation of reporter gene activity (Figure 17), over fifty putative interactions were tested by yeast two hybrid analysis.

To characterize the Chs3 domain interacting with the activator Chs4, the C1, C3, C4 and C7 domains of Chs3 were tested for their ability to bind Chs4. The previously reported interaction between Chs3 and Chs4 was confirmed and the C3 domain (amino acid positions 226-452) was identified as the binding region to Chs4 (Figure 17; DeMarini *et al*., 1997; Ono *et al*., 2000). In contrast to the C3 domain, no additional interaction between other Chs3 domains and Chs4 were detected (not shown).

Subsequently, the C1, C3, C4 and C7 domains of Chs3 were tested for their ability to interact with the cytosolic S2, S5 and S8 domains of Ste24 (Tam *et al*., 2001).

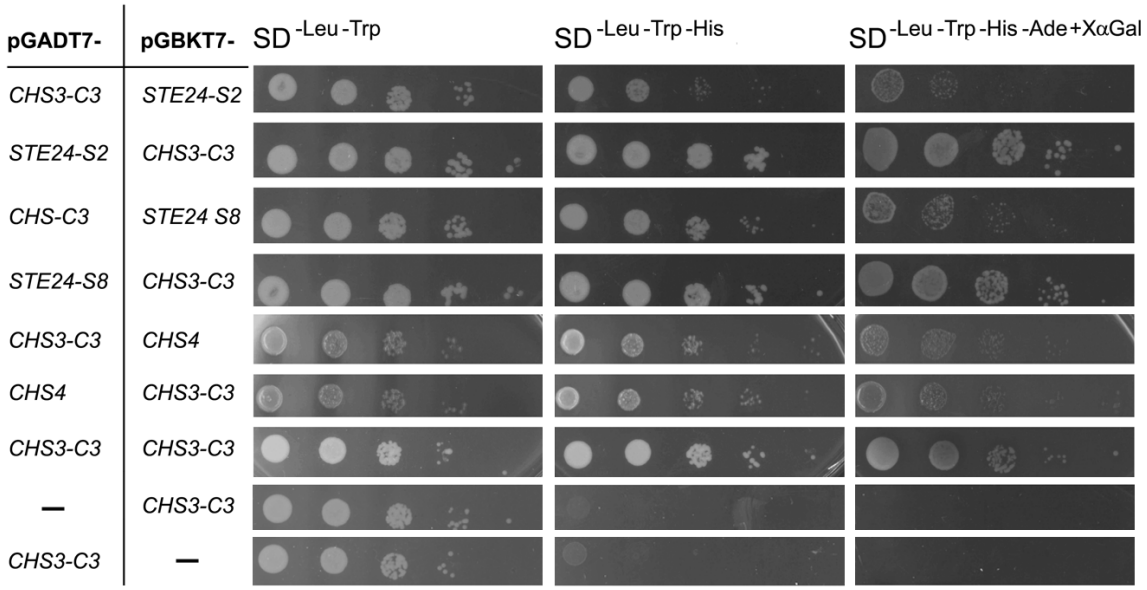


Figure 17. Yeast two-hybrid analysis to identify interacting domains – AH109 cells were co-transformed with bait and pray vectors. Cells grown overnight in liquid SD $^{-Leu\ -Trp}$ medium were diluted with water to a final concentration of 1 x 10^7 cells/ml. Five µl of each suspension and three subsequent 10-fold serial dilutions were individually spotted onto SD$^{-Leu\ -Trp}$, SD$^{-Leu\ -Trp\ -His}$ and SD$^{-Leu\ -Trp\ -His\ -Ade\ +X\alpha Gal}$ plates for selection. Cells were incubated at 30°C for 2 days.

The result in Figure 17 show that the C3 domain of Chs3 interacts with an interface of Ste24 formed at the cytoplasmic side by the S2 and S8 domains. The observation that the C3 domain of Chs3 interacts with regions of proteins known to be exposed to the cytoplasm strongly supports a topology model placing this domain at the cytoplasmic site of the membrane (Figure 17).

Chitin synthases from insects have been recently reported to exist as oligomeric complexes (Maue *et al*., 2009). To analyze putative oligomerization with the yeast two hybrid system, the soluble domains of Chs3 were mutually tested for interactions. Indeed, a binding between two C3 domains could be observed (Figure 17). None of the other Chs3, Ste24 or Chs4 domains displayed significant mutual or self interactions. No interaction could be detected (data not shown).

3.3.2 Chitin synthesis in *ste24*Δ cells and other mutants

3.3.2.1 Ste24 affects CFW-resistance

The yeast two hybrid analyses in chapter 3.3.1 confirmed the interaction between the Ste24 and the Chs3. But does Ste24 also have an impact on chitin synthesis? If Ste24 modulates the chitin synthase, the deletion or overexpression of Ste24 should have an impact on chitin levels. To test whether Ste24 affects chitin levels and hence might affect Chs3 activity, wild-type and different mutant strains were analyzed for their sensitivity towards CFW. As reported previously, wild-type cells were highly sensitive towards CFW, while *chs3*Δ or *chs4*Δ cells exhibited significant resistance (Figures 18, 19). When *ste24*Δ cells were analyzed for CFW sensitivity, a clear, but moderate resistance towards CFW was observed. Cell growth of *ste24*Δ in present of CFW was notably stronger than for wild-type cells, whereas *chs3*Δ or *chs4*Δ mutants were even more resistant to CFW (Figure 18). To analyse whether the observed effects on chitin levels are specific for Ste24, *rce1*Δ cells were examined for CFW sensitivity, a mutant lacking the other known CaaX-protease in yeast. In contrast to *ste24*Δ cells, *rce1*Δ and *ste14*Δ cells were highly sensitive to CFW, comparable to the sensitivity of wild-type cells (Figure 18). Cells overexpressing *STE24* were also tested for CFW sensitivity. For this purpose, the ORF of Ste24 was cloned into the multicopy vector pJJH71, placing it under the control of the constitutive promoter PFK2 (vector map see Addendix). After transformation of wild-type cells the CFW phenotype was monitored. As expected, the, overexpression of *STE24* lead to hypersensitivity

towards CFW (Figure 18). These observations strongly indicate that modulation of *ste24* expression affects chitin levels.

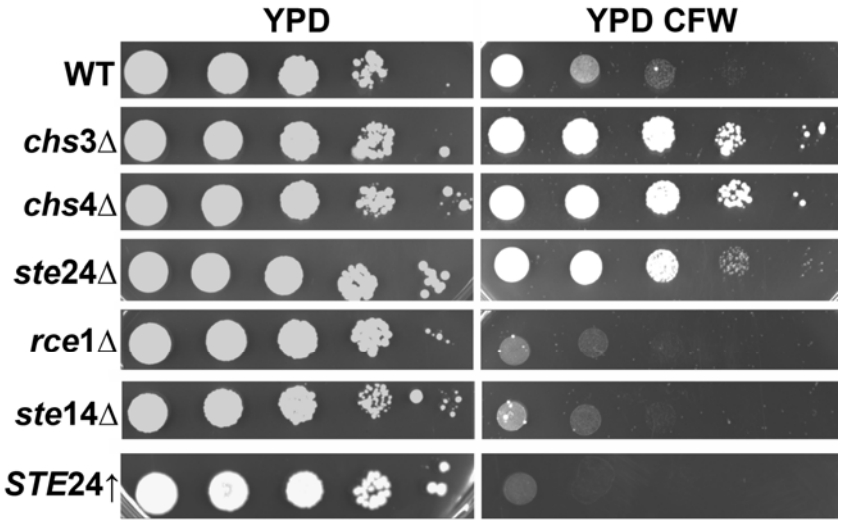


Figure 18. CFW resistance phenotypes in different yeast strains - Wild-type, *chs3*Δ, *chs4*Δ, *ste24*Δ, *rce1*Δ and *STE24*↑cells were grown overnight in liquid full medium and diluted with water to a final concentration of 1 x 10^7 cells/ml. Five µl of each suspension and three subsequent 10-fold serial dilutions were spotted onto YPD media plates with or without 50 µg/ml CFW. Cells were incubated at 30°C for 3 days.

3.3.2.2 Ste24 affects chitin levels

To determine relative chitin amounts in the cell wall of different yeast strains, a CFW fluorescence assay based on the specific binding of CFW was performed to estimate relative chitin levels in the cell wall (Figure 19). Compared to wild-type cells the observed total CFW fluorescence of *chs3*Δ was decreased approximately 70%. The difference in CFW fluorescence between wild-type and *chs3*Δ was set to 100% to represent chitin synthesis mediated by Chs3. Consistently with the results from our CFW growth tests, *ste24*Δ cells showed significantly less CFW fluorescence and hence reduced chitin levels (78.0% ± 1.8%, n=20). *STE24* overexpressing cells showed a slightly higher CFW fluorescence and hence increased chitin levels (110.0% ± 2.6%, n=15). Cells that were defective in *CHS4* encoding an activator of Chs3, displayed a very low CFW fluorescence which was comparable to that of *chs3*Δ cells (2.0% ± 1.4%, n=20). This method was previously published by Lam *et al*. (2006), but only for characterization of chitin-deficient mutants. This was the first time that

a method based on fluorescence generated by binding of CFW to chitin, is used to measure quantitatively the amount of chitin in the cells. To evaluate the results from the CFW fluorescence assay, we measured chitin amounts independently by the method of Morgan-Elson (Figure 18). This method is based on specific digestion of cell chitin by chitinase followed by colorimetric determination of GlcNAc amounts. Wild-type cells exhibited a chitin content of 5.2 nmol GlcNAc/mg cells (± 0.2, n=6), which was significantly reduced to 1.8 nmol GlcNAc/mg cells (± 0.1, n=6) in *chs3*Δ cells.

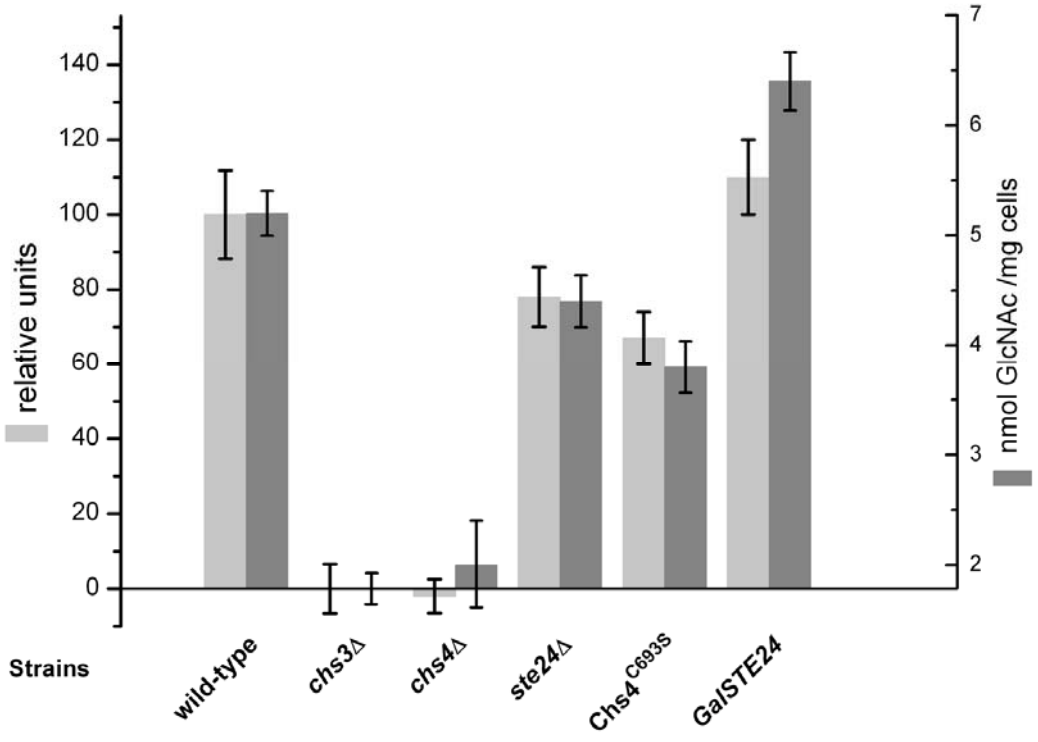


Figure 19. Chitin amounts in yeast cells defective in different genes – Quantification of chitin levels. Wild-type (n=40), *ste24*Δ (n=40), *chs3*Δ (n=20), *chs4*Δ (n=20), Chs4^{C693S} (n=20) and *STE24*↑ (n=15) cells (10^9/ml) were grown for 2 days on YPD or YPG plates containing 50 µg/ml CFW. CFW fluorescence was excited by UV-light and fluorescence was quantified densitometrically (light gray bars). Relative CFW fluorescence is given in percent as mean values of deviations from wild-type cells (± S.E.). Chitin amounts were additionally quantified by the Morgan-Elson method (dark gray bars). Data represent averages (± S.E.) obtained from six independent experiments for each yeast strain. One-Way ANOVA and Tukey HSD test revealed significant differences for all variations from wild-type cells (HSD 0.05 = 7.57; HSD 0.01 = 9.08 for the CFW assay and HSD 0.05 = 0.44; HSD 0.01 = 0.54 for the Morgan-Elson assay).

Both values are in good agreement with previous measurements (Bulik *et al*., 2003). In line with the CFW fluorescence assay the chitin content in *ste24*Δ was

reduced to 4.4 nmol GlcNAc/mg cells (± 0.2, n=6), which is about 76% of the chitin produced by Chs3. As observed in the CFW fluorescence assay, *STE24* overexpressing cells increased chitin levels of 6.4 GlcNAc/mg cells (± 0.3, n=6), which corresponds to 135% of the chitin produced by Chs3 and is somewhat higher than estimated from CFW fluorescence measurements. The chitin content of *chs4*Δ cells was with 2.0 nmol GlcNAc/mg cells (± 0.3, n=6) in the same range as that of *chs3*Δ cells. Overall, the chemical determination of chitin content was in good agreement with the estimate based on CFW fluorescence. In all experiments chitin level are decreased when *ste24* is deleted, whereas it is increased when Ste24 is overexpressed.

3.3.2.3 Ste24 does not affects cellular distribution of chitin

To visualize chitin deposition in growing wild-type, *ste24*Δ, *chs3*Δ and *STE24*↑ cells, cells were stained with CFW and monitored under a fluorescence microscope. As expected, wild-type cell show an intensive fluorescence signal at the bud neck and a weaker one throughout the cell wall. In contrast, fluorescence of *chs3*Δ mutant was almost depleted. The fluorescence in CFW stained *ste24*Δ cells was notably weaker than in wild-type cells, whereas cells overexpressing *STE24* had the most intensive signal (Figure 20A). These results are in line with the results shown in Figure 19. Like in wild-type cells, *ste24*Δ and *STE24*↑ cells chitin localized at the bud neck and throughout the cell wall. To test if the chitin distribution in *ste24*Δ and *STE24*↑ cells is decreased overall or just at the bud neck, the local fluorescence intensities at the bud neck and in the cell walls were measured. The ratios of local fluorescence intensities between bud and cell wall were determined for individual cells, and mean values of fluorescence intensity ratios of wild-type and mutant cells were calculated. The fluorescence intensity ratios were 1.7 (± 0.3, n=10) in wild-type, 1.5 (± 0.5, n=8) in *chs3*Δ, 1.4 (± 0.2, n=9) in *ste24*Δ and 2.0 (± 0.5, n=10) in *STE24*↑ cells. Thus, although chitin content in various mutant cells differed significantly from that of wild-type cells, its distribution did

not change significantly as indicated by ANOVA tests (HSD 0.05 = 0.50; HSD 0.01 = 0.63) of the combined data (Figure 20B).

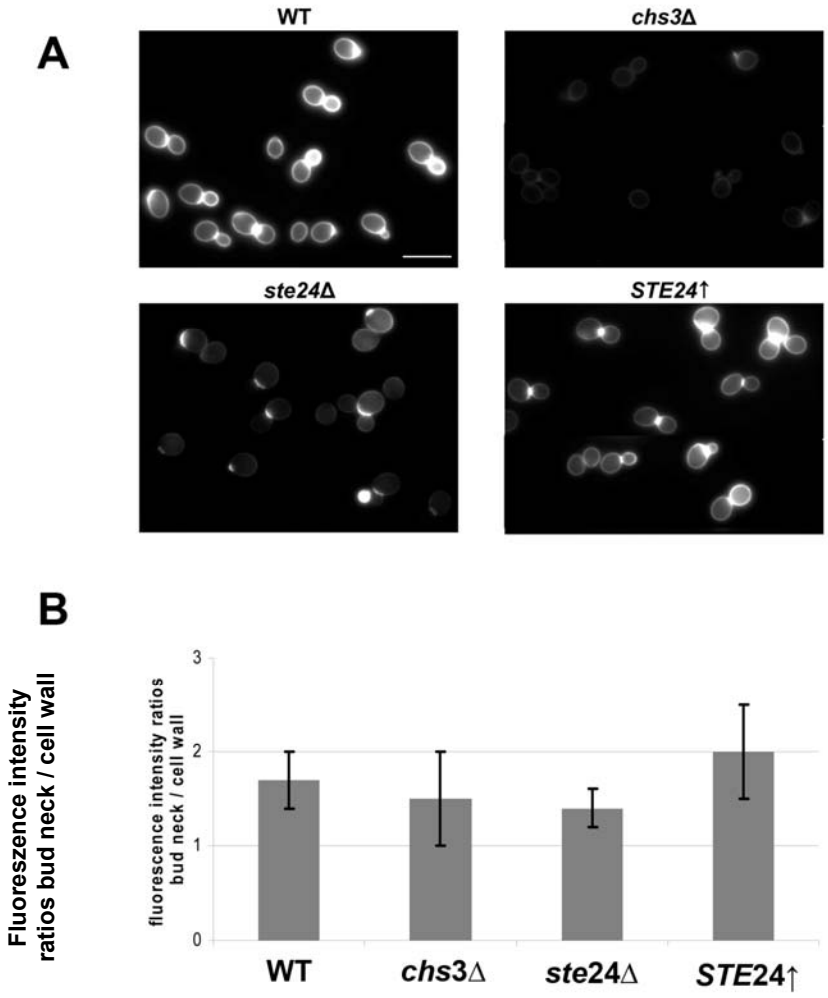


Figure 20. Chitin deposition in yeast cells defective in different genes – (A) Micrographs of wild-type, *ste24*Δ, *chs3*Δ, and *STE24*↑cells. Cells were grown overnight in liquid YPD or YPG medium and stained with 0.02% CFW. Scale bar, 10 µm. **(B)** Fluorescence intensity ratios (bud neck / cell wall) Wild-type (n=10), *chs3*Δ (n=8), *ste24*Δ (n=9) and *STE24*↑ (n=10). One-Way ANOVA and Tukey HSD test revealed no significant differences for all variations from wild-type cells (HSD 0.05 = 0.50; HSD 0.01 = 0.63).

3.3.3 Catalytic activity of Ste24

3.3.3.1 Catalytically inactive Ste24 does not restore CFW sensitivity in *ste24*Δ cells

To examine whether the observed effects on chitin levels depend on the catalytic activity of Ste24, a catalytically inactive mutant Ste24^{E298G} was generated and tested whether it can restore CFW sensitivity in *ste24*Δ cells. After cloning *STE24* into a centromeric vector and placing it under the control of its endogenous promoter, the catalytic glutamate within the conserved HExxH motif was changed into a glycine residue by site directed mutagenesis. Next, *ste24*Δ and *ste24*Δ *rce1*Δ *MAT***a** cells were transformed with the Ste24^{E298G} vector. As positive control *ste24*Δ and *ste24*Δ *rce1*Δ *MAT***a** cells were transformed with the *STE24* wild-type vector. *MAT***a** cells carrying mutations in this motif are unable to process the MF**a** precursor and hence are mating-deficient (Fujimura-Kamada *et al*., 1997). To assess whether a mutant expressing a catalytically inactive form of Ste24 had been successfully generated, a halo assay as described by Trueblood *et al.* (2000) was performed. In this assay, the growth of MATα cells is suppressed when active MF**a** is produced by cleavage of the MF**a** precursor by active Ste24 producing a halo. The halo will be absent if Ste24 is not active. As *rce1*Δ and *ste24*Δ single mutants still exhibit a halo due to the activity of the remaining CaaX protease, they are almost indistinguishable from wild-type cells (Figure 21B). However, in *rce1*Δ *ste24*Δ double deletion mutants, no halos were visible. When *rce1*Δ *ste24*Δ double deletion mutants were transformed with a vector carrying *STE24*, the halo phenotype of wild-type cells was restored. In contrast, transformation of *ste24*Δ cells with a vector carrying *ste24^{E298G}* showed no halo, indicating that the catalytic activity of Ste24 was abolished (Figure 21B). Next, CFW resistance in *ste24*Δ cells expressing either *STE24* or the catalytically inactive *ste24^{E298G}* was tested. As shown in Figure 21C transformation with the wild-type *STE24* vector restored CFW sensitivity of *ste24*Δ cells, while transformation with the catalytically inactive *ste24^{E298G}* vector did not. Thus, the

observed effect on chitin synthesis in *ste*24 mutants depends on the catalytic activity of Ste24.

3.3.3.2 Human and insect homologues of Ste24 restore CFW sensitivity in *ste24*Δ cells

Ste24 is a conserved CaaX protease and homologues can be found ubiquitously in eukaryotes. Despite a amino acid sequence identity of about 36% between yeast Ste24 and its human homologue ZmpSte24 (Figure 21A), Leung *et al*. (2001) demonstrated that human ZmpSte24 can restore the halo phenotype in the *rce1*Δ *ste24*Δ cells. To test whether *ZmpSTE24* and the insect *STE24* homologue from the red flour beetle *Tribolium castaneum* (*TcSTE24*) fully restore the halo phenotype in the *rce1*Δ *ste24*Δ cells and the CFW sensitivity in *ste24*Δ cells, the cDNAs from the *STE24* homologues were amplified from human cDNA or *T.castaneum* cDNA and cloned into the multi-copy vector pJJH71 and placing it under the control of the constitutive promoter PFK2 (vector map see Appendix). Next, *ste24*Δ and *rce1*Δ *ste24*Δ were transformed with the *ZmpSTE24* and *TcSTE24* vectors respectively. As shown in Figure 21B not only human *ZmpSTE24* but also the insect *TcSTE24* homologue fully restored the halo phenotype in the *rce1*Δ *ste24*Δ cells. To test if the human and insect *STE24* homologues can restore also the CFW phenotype, *ZmpSTE24* and *TcSTE*24 were expressed in *ste24*Δ cells and plated on CFW containing plates. The growth tests shown in Figure 21C indicate that *ste24*Δ cells expressing human or *Tribolium STE*24 exhibit the same sensitivity to CFW as wild-type cells, suggesting that they are functional orthologues of yeast *STE24*.

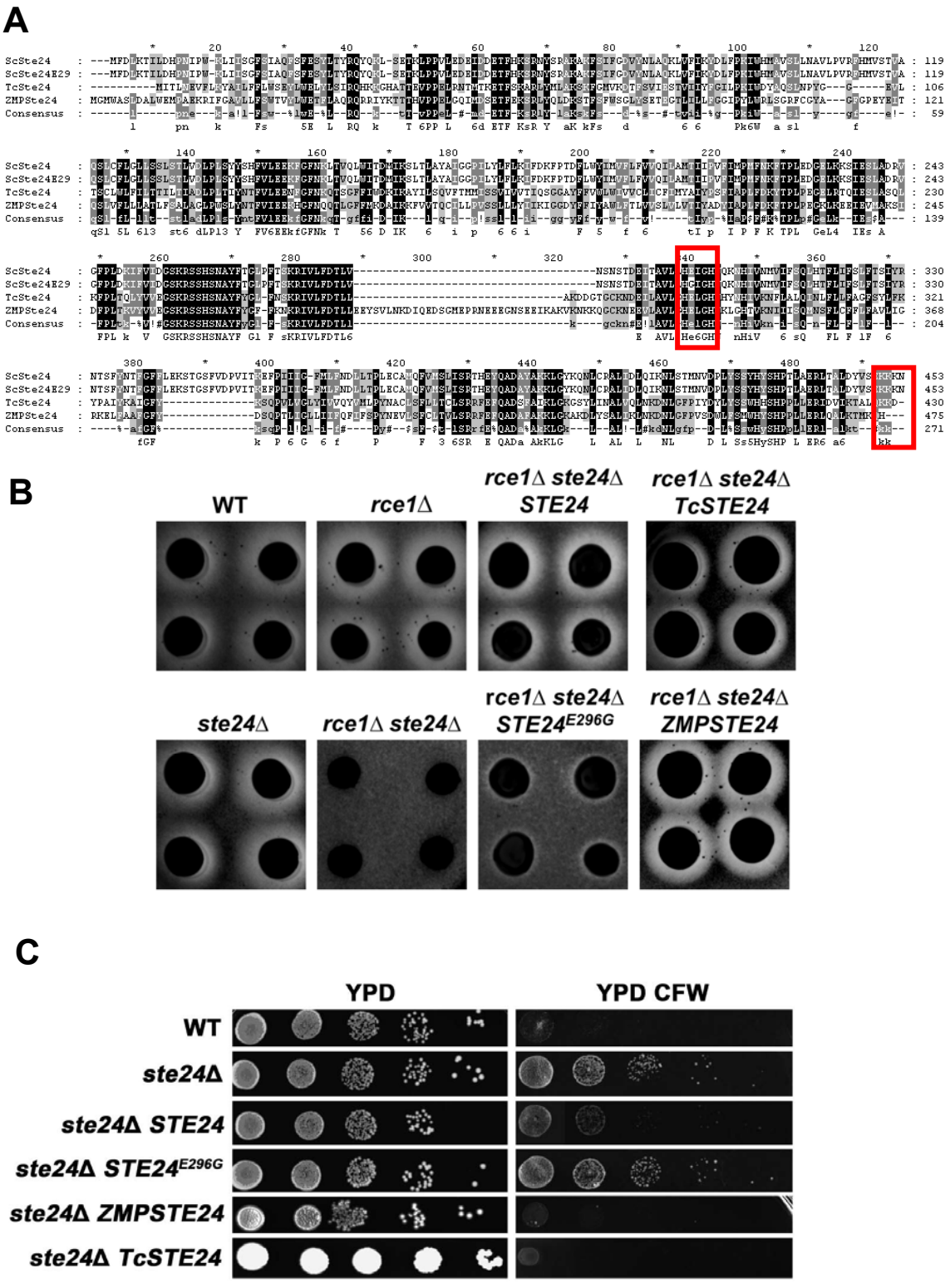


Figure 21. Catalytic active, inactive Ste24, ZmpSte24 and TcSte24 - (A) ClustalW amino acid sequence alignment of Ste24, Ste24^{E298G}, ZmpSte24 and TcSte24. The catalytic HExxH motif and the C-terminal KKKN motif are highlighted in a red box.**(B)** Halo assay with catalytic active and inactive Ste24 - Different MAT**a** cells (wild-type, *ste24*Δ, *rce1*Δ, *ste24*Δ *rce1*Δ, *ste24*Δ *rce1*Δ *pRS415ste24*, *ste24*Δ *rce1*Δ *pRS415ste24*^{E296G}, *ste24*Δ *rce1*Δ pJJH71-*ZmpSTE24* and *ste24*Δ *rce1*Δ pJJH71-*TcSTE24*) were grown overnight in liquid YPD medium and adjusted to a final concentration of 5 × 10^9 cells/ml. Then, 1.5 µl of each suspension was

spotted onto YPD media at 42°C containing 0.04% Triton X-100 and 2 × 10^6 cells of the MATα *sst2*Δ strain cells. Cells were incubated at 30°C for 3 days. Gray scales were inverted for a better visualization of the halos. **(C)** Restoration of CFW sensitivity. Wild-type, *ste24*Δ, *ste24*Δ *pRS415ste24,* *ste24*Δ *pRS415ste24*^{E296G}, *ste24*Δ *pJJH71-ZmpSTE24* and *ste24*Δ *pJJH71-TcSTE24* cells were grown overnight in liquid YPD medium and diluted to a final concentration of 1 x 10^7 cells/ml in water. 5 µl of each suspension and three subsequent 10-fold serial dilutions were spotted onto YPD plates with or without 50 µg/ml CFW. Cells were incubated at 30°C for 3 days.

3.3.4 Chs4 is a substrate of Ste24 influencing chitin synthesis

3.3.4.1 Deletion of *ste24* in *MAT*α affects CFW resistance

MF**a** is the only substrate so far described for Ste24. However, could the insufficient processing of MF**a** leading to sterile yeast cell (Figure 21B) also explain on the effects on chitin synthesis under vegetative growth conditions? If insufficient processing of MF**a** would affect chitin synthesis, only the chitin synthesis in *ste24*Δ *MAT**a*** cells would be affected, since *MAT*α cells do not express MFa. Therefore, *MAT**a*** wild-type, *MAT**a*** *ste24*Δ, *MAT*α wild-type and *MAT*α *ste24*Δ cells were examined for CFW resistance. The results depicted in Figure 22 clearly show that the deletion of ste24 in *MAT*α cells also leads to an increase in CFW resistance. These results suggest that MF**a** processing by Ste24 do not modulate chitin synthesis in vegetative cells.

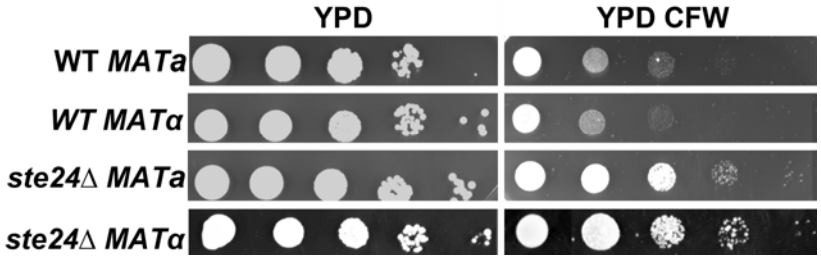


Figure 22. CFW phenotypes of different yeast strains – *MAT**a*** wild-type, *MAT**a*** *ste24*Δ, *MAT*α wild-type and *MAT*α *ste24*Δ cells were grown overnight in liquid full medium and diluted with water to a final concentration of 1 x 10^7 cells/ml. Five µl of each suspension and three subsequent 10-fold serial dilutions were spotted onto YPD media plates with or without 50 µg/ml CFW. Cells were incubated at 30°C for 3 days.

3.3.4.2 Immunoblots do not support that Chs3 is a substrate for Ste24

The results shown in Figure 22 demonstrated that cleavage of MF**a** is not responsible for modulating chitin synthesis under vegetative conditions. But

what could be the substrate for Ste24 mediating the effects on chitin synthesis? Ste24 has dual roles in protein processing. In addition to its function of cleaving the C-terminal CaaX motif, it is also known to cleave the N-terminus of the MF**a** precursor (Trueblood *et al*., 2000). Since Chs3 lacks a C-terminal CaaX motif, Ste24 obviously does not cleave the C-terminus of Chs3. To test whether Ste24 cleaves Chs3 at all, the ORFs of Chs3 and 13Myc were cloned into the centromeric plasmids pAG503 and placing it under the control of its endogenous CHS3 promoter. Then wild-type and *ste24*Δ were transformed with the Chs3-13Myc (C-terminal Myc tag) vector. Finally, total cellular extracts from wild-type and *ste24*Δ cells expressing Chs3-13Myc from centromeric plasmids were prepared, the proteins were separated by SDS-PAGE and immuno-blots were performed with anti-Myc antibodies. In both cell extracts, two protein bands of approximately 100 kDa and 160 kDa could be detect, which both did not shift in response to deletion of *ste24* (Figure 23). This result suggests that Chs3 is not cleaved by Ste24 and further substantiates the findings of Cos *et al*. (1998) who also observed no differences in the migration behavior of Chs3 tagged with HA-epitopes either at the N- or C-terminus. However, the possibility that a small peptide is cleaved off by Ste24 at the N- or C-terminus can not totally be excluded, in case the tags interfere with the cleavage sites close to the N- or C-terminal ends.

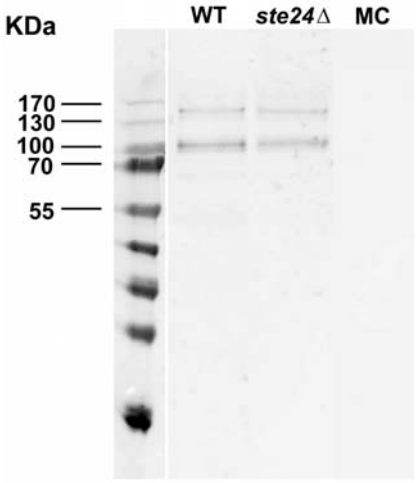


Figure 23. Immunoblot detecting Chs3-13Myc in transformed wild-type and *ste24*Δ cell extracts - Total cellular extracts obtained from wild-type (WT) and *ste24*Δ cells expressing

Chs3-13Myc from centromeric plasmids were subjected to SDS-PAGE and subsequently blotted onto nitrocellulose membranes for immunostaining with anti-cMyc antibodies. Standard proteins are indicated with molecular masses given in kDa. MC, mock control using wild-type yeast cell transformed with the empty pRS415 vector.

3.3.4.3 Prenylation of Chs4 is required for Ste24 effects on chitin synthesis

The results described above imply that the proteolytic activity of Ste24 is required to maintain wild-type chitin levels. Although Chs3 binds to Ste24, direct processing of Chs3 by the CaaX protease turned out to be unlikely. However, Ste24 may process another protein required for chitin synthesis. An obvious candidate is Chs4, a known activator of Chs3 (DeMarini *et al*., 1997; Trilla *et al*., 1997), which unlike to Chs3 possesses a C-terminal CaaX motive (CVIM). Thus, Chs4 fulfils all sequence requirements to be cleaved by Ste24 (Trueblood *et al*., 2000). Furthermore, Chs4 is known to be prenylated, which is a prerequisite for removal of the C-terminal tri-peptide by the CaaX protease. Direct biochemical proof corroborating that Chs4 is a substrate of Ste24 is difficult to obtain, as protein analysis is hampered by prenylation and the fact that only a tripeptide is removed yielding only small differences in molecular masses. Therefore, a genetic approach was used to address this issue based on the observation that mutants expressing a non-prenylated version of Chs4, in which the cysteine of the CaaX motif is replaced by a serine ($Chs4^{C693S}$), exhibits a CFW resistance phenotype (Grabinska *et al*., 2007). The reported CFW resistance phenotype of $Chs4^{C693S}$ could be confirmed in this study (Figure 24B). CFW resistance of *chs4*^{C693S} cells was more pronounced than that of *ste24*Δ cells but less pronounced than that of *chs4*Δ cells (Figure 24B). The chitin content in *chs4*^{C693S} cells is reduced by about 33%, about 11% more than the reduction of the chitin content in *ste24*Δ cells (Figure 19). If Ste24 mediated cleavage of the CaaX motif of Chs4 is required for Chs3 activity, the CFW phenotype of non-prenylated Chs4 cells should be unchanged when *ste24* is overexpressed or deleted. As shown in Figure 24B, $Chs4^{C693S}$ cells exhibit the same CFW phenotype as *ste24*Δ $Chs4^{C693S}$ or *STE24* overexpressing $Chs4^{C693S}$ cells.

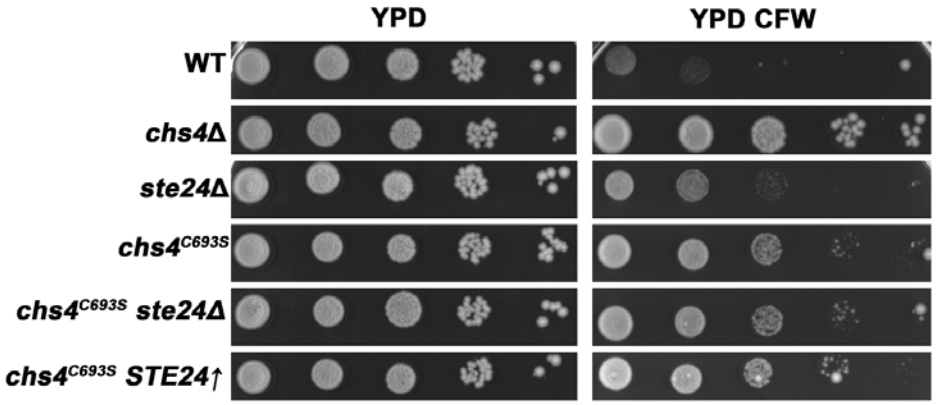


Figure 24. CFW resistance phenotypes of yeast cells defective in wild-type, *chs4*^{C693S}, *ste24*Δ, *ste24*Δ *chs4*^{C693S}, *chs4*^{C693S} pJJH71-*STE24* cells – Cells were grown overnight in liquid YPD medium and diluted to a final concentration of 1 x 10^7 cells/ml in water. 5 µl of each suspension and three subsequent 10-fold serial dilutions were spotted onto YPD plates with or without 50 µg/ml CFW. Cells were incubated at 30°C for 3 days.

3.3.5 Localization of Chs3, Chs4 and Ste24 in different yeast mutants

3.3.5.1 Deletion of *chs3* or *chs4* has no influence on Ste24 localization

To investigate if Chs3 or Chs4 influences the intracellular localization of Ste24, Ste24-GFP localization was monitored by fluorescence microscopy in wild-type, *chs3*Δ and *chs4*Δ backgrounds. As shown Figure 25, Ste24-GFP localizes at the ER membrane in wild-type, *chs3*Δ and *chs4*Δ cells. These results clearly demonstrate that localization of Ste24 at the ER is independent from Chs3 or Chs4.

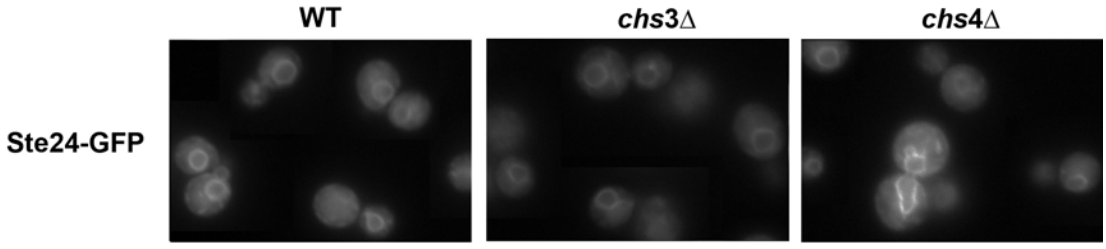


Figure 25. Intracellular localization of Ste24-GFP in wild-type, *chs3*Δ and *chs4*Δ cells – Cells were observed in the early logarithmic growth phase. Ste24-GFP was visualized by fluorescence microscopy.

3.3.5.2 Membrane association of Chs4 requires prenylation but is independent of further CaaX processing

To investigate the contribution of prenylation and cleavage of the CaaX motif independently, GFP- Chs4 and GFP-Chs4^{C693S} were expressed in wild-type, *ste24*Δ and *ste24*Δ *rce1*Δ cells. In order to observe the localization of Chs4 properly, Chs4 was N-terminal tagged with GFP and and cloned into the centromeric vector pGREG575. A non-prenylation Chs4 mutant was cloned by mutating the cysteine within the CaaX sequence into serine. Next, wild-type, *ste24*Δ and *ste24*Δ *rce1*Δ cells were transformed with pGREG575-Chs4 and pGREG575-Chs4^{C693S} respectively. The intracellular distribution of GFP-Chs4 and GFP-Chs4^{C693S} was analyzed for each strain in at least 200 cells (Figure 26B). As shown in Figure 26, GFP-Chs4 is mainly membrane associated in wild-type cells. In over 98% of the analyzed wild-type cells GFP-Chs4 localizes at the plasma membrane. Likewise GFP-Chs4 localizes at the vacuolar and ER membranes in the vast majority of wild-type cells. In almost 60% of the wild-type cells GFP-Chs4 was visible at the bud neck. In *ste24*Δ or *ste24*Δ *rce1*Δ cells GFP-Chs4 localization was similar to wild-type cells (96% / 97% plasma membrane; 83% / 90% vacuole membrane; 72% / 75% ER and 52% / 53% bud neck, respectively). No significant differences in GFP-Chs4 localization was detectable in wild-type, *ste24*Δ or *ste24*Δ *rce1*Δ, suggesting that Ste24-mediated cleavage of the CaaX motif is not required for membrane association of Chs4. However, GFP-Chs4^{C693S} showed striking differences in localization compared to wild-type GFP-Chs4. The vast majority of GFP-Chs4^{C693S} localized in the cytoplasm (97% and no signal could be observed at the plasma membrane; Figure 26). In addition, in 80% of the analyzed cells GFP-Chs4^{C693S} localized in the nucleus. These results are in good agreement with results published by Reyes *et al*. (2007). The deletion of the CaaX proteases had, as expected, no influence on Chs4^{C693S}-GFP localization. Hence, membrane association of Chs4 depends on prenylation but not on subsequent steps of CaaX processing.

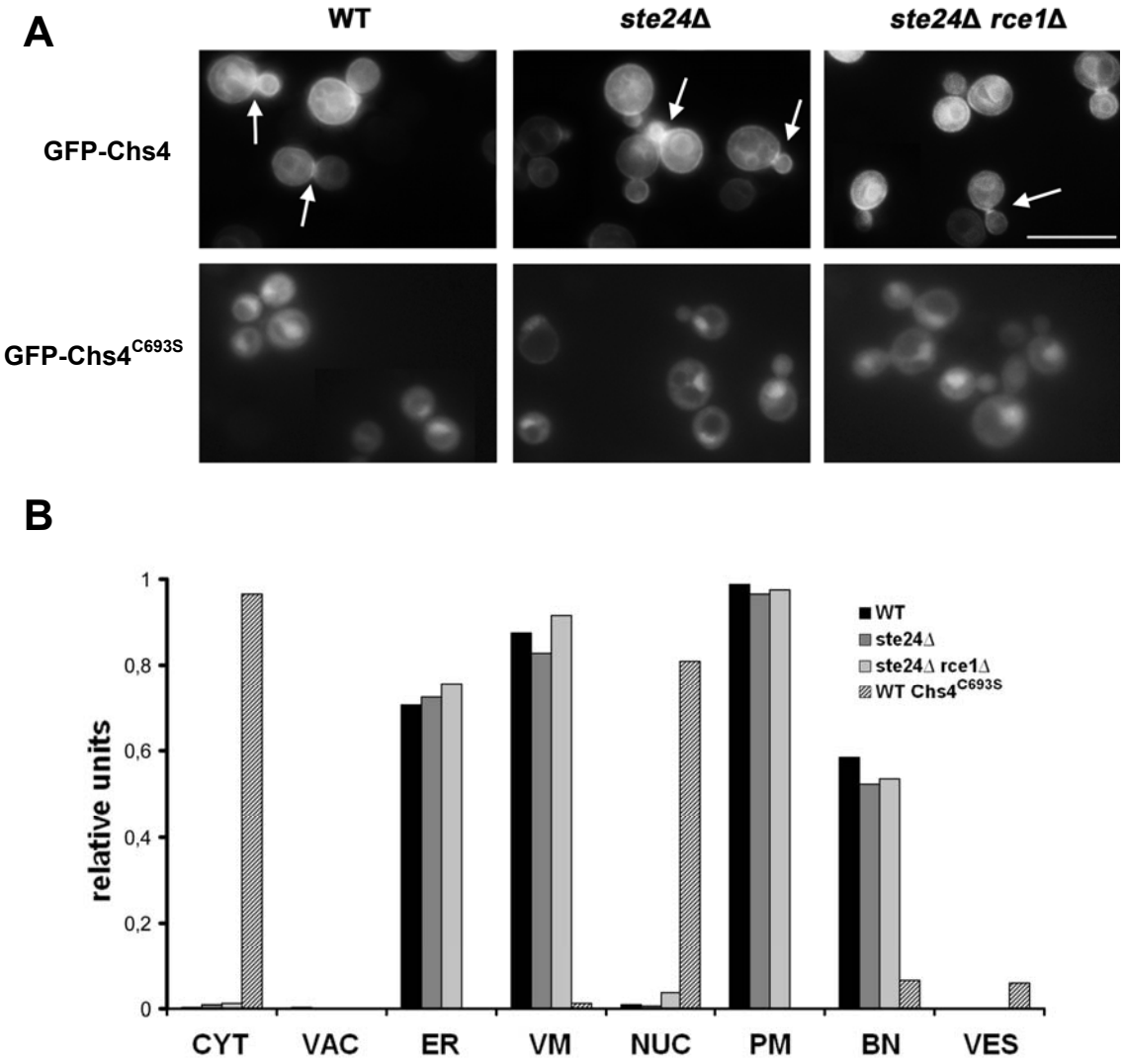


Figure 26. Intracellular localization of GFP-Chs4 and GFP-Chs4^{C693S} in wild-type, *ste24*Δ and *ste24*Δ *rce1*Δ cells – (A) Micrographs of GFP-Chs4 and GFP-Chs4^{C693S} in wild-type, *ste24*Δ and *ste24*Δ *rce1*Δ cells. Cells were grown overnight in liquid media containing 2% raffinose and were observed 3 hours after adding 2% galactose to initiate expression of GFP-Chs4 or GFP-Chs4^{C693S}. GFP-Chs4 and GFP-Chs4^{C693S} were localized by fluorescence microscopy. Scale bar, 10 μm. **(B)** Quantitative analysis of intracellular distribution of GFP-Chs4 in wild-type, *ste24*Δ and *ste24*Δ *rce1*Δ and GFP-Chs4^{C693S} cells - Cells were grown overnight in liquid media containing 2% raffinose and were observed 3 hours after adding 2% galactose to initiate expression of GFP-Chs4 or GFP-Chs4^{C693S}. GFP-Chs4 and GFP-Chs4^{C693S} were visualized by fluorescence microscopy. Protein distribution was analyzed in 150-250 single cells for each strain. CYT, cytoplasm; VAC, vacuole; ER, endoplasmic reticulum; VM, vacuolar membrane; NUC, nucleus; PM, plasma membrane; BN, bud neck; VES, vesicle.

3.3.5.3 Deletion of *ste24* leads to reduced levels of Chs3 at the bud neck

To address the question, whether the differences in chitin levels result from Ste24-mediated perturbations of the intracellular transport of Chs3, the localization of Chs3-GFP was analyzed, after demonstrating that Chs3-GFP is fully functional, as it can restore the wild-type CFW phenotype in *chs3*Δ cells (Figure 27).

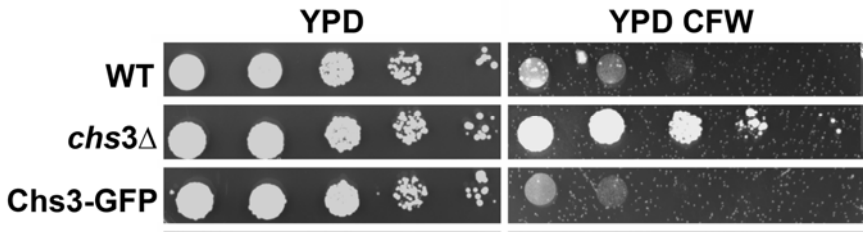


Figure 27. CFW resistance phenotypes of yeast cells defective in different genes - Wild-type, *chs3*Δ, Chs3-GFP cells were grown overnight in liquid YPD medium and diluted to a final concentration of 1 x 10^7 cells/ml in water. 5 µl of each suspension and three subsequent 10-fold serial dilutions were spotted onto YPD plates with or without 50 µg/ml CFW. Cells were incubated at 30°C for 3 days.

Intracellular distribution of Chs3-GFP in wild-type, *ste24*Δ and *chs4*Δ background was quantitatively analyzed. In wild-type cells, Chs3-GFP localizes to vesicles (about 76% of examined cells; Figure 28). Chs3-GFP was visible at the bud in 38% and at the plasma membrane in 7% of all wild-type cells, in cells overexpressing *STE24*↑, intracellular distribution of Chs3-GFP was similar to its distribution in wild-type cells (82% vesicles; 38% bud and 11% plasma membrane). Like in wild-type cells, Chs3-GFP localized mainly to vesicles in *ste24*Δ cells (81%). However, in *ste24*Δ cells the localization of Chs3-GFP at the bud neck was significantly reduced by 50% compared to wild-type cells (14%; Figure 28). In *chs4*Δ cells the Chs3-GFP signal was more diffuse than in the other examined strains and Chs3-GFP was less frequently at the bud neck as in the case of *ste24*Δ cells (approximately 20% of all examined cells). Thus, the data indicate that the effects of *ste24* on chitin synthesis are mediated by delocalization of Chs3.

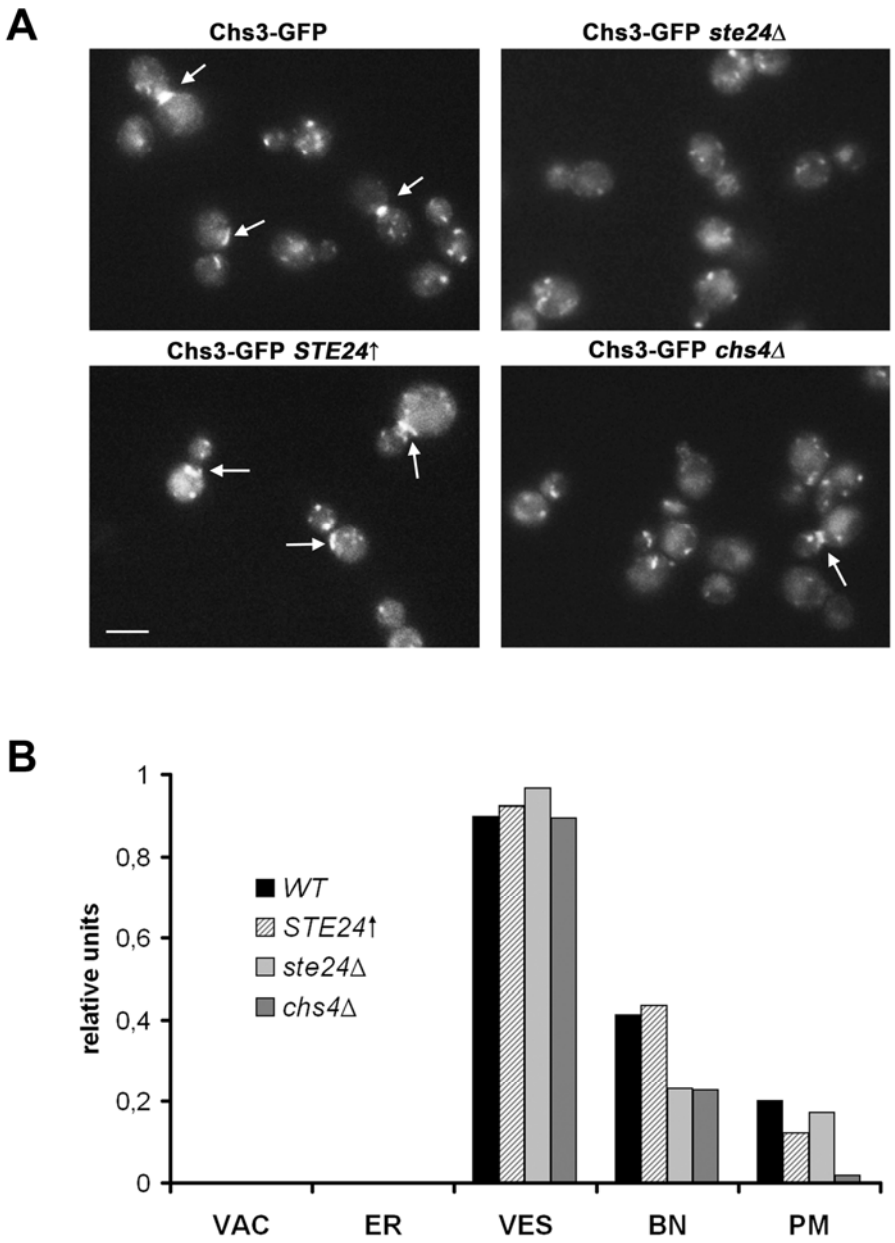


Figure 28. Intracellular localization of Chs3-GFP in wild-type, *STE24*↑, *ste24*Δ and *chs4*Δ cells – (A) Fluorescence microscopy. Cells were observed in the early logarithmic growth phase. Chs3-GFP was visualized by fluorescence microscopy. Arrows point to increased fluorescence at the bud necks. Scale bar, 5 µm. **(B)** Quantitative analysis of changes in Chs3-GFP localization. The distribution of Chs3-GFP was analyzed in wild-type, *ste24*Δ and *ste24*Δ *rce1*Δ cells (150-250 single cells each strain). VAC, vacuole; ER, endoplasmic reticulum; PM, plasma membrane; BN, bud neck; VES, vesicle.

4 Discussion

4.1 Regulation of chitin synthesis

Regulation of chitin synthesis is particularly important. Chitin is synthesized in fungi at different stages of the cell cycle and embedded in different parts of the cell wall (Chuang and Schekman, 1996). Furthermore, in animals chitin is synthesized in specific tissues depending on the developmental stage (Merzendorfer, 2006; Merzendorfer and Zimoch, 2003). Interference with chitin synthesis or deletion of chitin synthases lead to severe moulting defects and morphological abnormalities. A mutation in the *CHS1* gene of *D. melanogaster* leads to deformation of the head cuticle during the embryogenesis. This phenotype was eponymous for the *D. melanogaster* gene name *krotzkopf verkert* (*kkv*; Ostrowski *et al*., 2002). In Yeast mutants defective in *chs2* only grow in microcolonies, because of the failure to separate from their mothers cells (Nagahashi *et al*., 1995), whereas *chs1* or *chs3* deletion mutants only exhibit a weak phenotype (Valdivieso *et al*., 1991).

4.1.1 Transcriptional regulation

Chitin synthases are tightly regulated membrane proteins. In insects, tissue specific chitin synthesis is regulated on the transcriptional level. Until know, all insects investigated carry two different chitin synthase genes, *CHS1* and *CHS2*. As reported for *M. sexta* and *T. castaneum*, *CHS1* is expressed in the epidermis and in trachea and produced the chitin which is deposited in the cuticle. *CHS2* on the other hand is expressed in the midgut epithelial cells stabilizing the structure of the peritrophic matrix (Arakane *et al*., 2005; Hogenkamp *et al*., 2005; Lehane, 1997; Zimoch *et al*., 2005). In addition to the distinct spatial expression, transcription of chitin synthases is also temporally regulated during larval development of *M. sexta*. For instance, *MsCHS1* expression is up-regulated during molt and down-regulated during intermolt, synthesizing chitin for a newly assembling cuticle, while *MsCHS2* expression is down-regulated

during molt and up-regulated during the intermolt, synthesizing chitin for the peritrophic matrix while the animal is feeding (Zimoch *et al*., 2005).

In yeast, transcription of the three *chs* genes is differentially regulated. While *CHS1* and *CHS3* are not regulated on the transcriptional level, *CHS2* is largely regulated by processes of synthesis and degradation of its protein (Choi *et al*., 1994). Studies in synchronized cultures revealed that at the *CHS2* mRNA and Chs2 activity, peaks already at the end of the mitose (Choi *et al*., 1994). Moreover Chs2 activity is rapidly decreased when transcription is abolished. Interestingly the level of Chs2 activity decreases despite the presence of relatively high levels of mRNA (Choi *et al*., 1994). Degradation of Chs4 is discussed in the next chapter.

4.1.2 Posttranslational regulation: Protein degradation

In addition to transcriptional regulation, chitin synthases are regulated at the posttranslational level (Choi *et al*., 1994; Lesage and Bussey, 2006; Merzendorfer, 2006). What are the advantages of posttranslational regulation, and how and by which means is the chitin synthase regulated? One main advantage of posttranslational coversustranscriptional regulation is that cells can react much faster to changes in the environment.

A simple way to regulated enzyme activity is to rapidly degrade the protein if its activity is not needed anymore. In the regulation of yeast chitin synthesis this regulatory mechanism is realized in the case of Chs2, which is required for the synthesis of the primary septum in the telophase and associated with the constriction of the actomyosin ring (Schmidt *et al*., 2002; Zhang *et al*., 2006). Time laps studies revealed that Chs2 localizes shortly before septum formation at the bud neck and immediately disappears after cell division; this process takes place in less than 8 min. (Choi *et al*., 1994; Roh *et al*., 2002; Zhang *et al*., 2006). Chs2 is then transported in an *end4*-dependent matter to the vacuole and degraded by the vacuolar protease, Pep4 (Chuang and Schekman, 1996). In contrast, the other two yeast chitin synthases Chs1 and Chs3 are not degraded in the vacuole and exhibit a much longer “life span” (Choi *et al*., 1994). One obvious disadvantage of regulating protein activity by degradation is

that the protein can not be reactivated if its activity is needed again; instead the cell has to synthesize the protein once again, making it rather energy inefficient.

4.1.3 Posttranslational regulation: Transport

Another way to regulate chitin synthesis is controlling the transport of the chitin synthase to the site of action, which is the apical membrane in insects and the plasma membrane at the bud neck or at the shmoo in yeast. In yeast it has been reported that Chs1 and Chs3 are transported not through the “classical pathway” for plasma membrane proteins (ER→ Golgi→ PM→ and retrograde finally to the vacuoles), but delivered, after passing the Golgi, to specialized vesicles called chitosomes (Chuang and Schekman, 1996). One experiment particularly points to the importance of posttranslational control of chitin synthases in yeast. When expression of *CHS3* is under the control of the *Gal1* promoter and cells are transferred from galactose-containing medium to glucose-containing medium, the level of Chs3 activity increased over a period of about 10 to 12 h, though transcription of *CHS3* was shut off (Choi *et al*., 1994). This finding additionally suggests that vacuolar degradation does not account for Chs3.

The storage of Chs3 in chitosomes allows, whenever necessary, rapid reaction to adjust the amount of chitin synthases in the cell membrane controlling the synthesis of new chitin polymers. Due to their characteristic physical properties, chitosomes can be purified by ultracentrifugation. Isolated chitosomes from related yeast cells of *Mucor rouxii* exhibit a typical diameter between 40-70 nm (Bartnicki-Garcia, 2006). Electron microscopic analyses revealed that after addition of UDP-GlcNAc to the chitosomes, fibrillar material was synthesized, which, after a certain incubation time, led to the disintegration of the vesicles (Bracker *et al*., 1976). Additionally Siemieniewicz *et al*. (2007) purified Chs1 containing chitosomes form *S. cerevisiae chs3*Δ cells. The purified vesicles exhibited a diameter of 85 to 120 nm. Interrestingly the isolated Chs1 chitosomes ejected tiny fibers upon addition of UDP-GluNAc. This reaction was inhibited by addition of chitin synthase inhibitor nikkomycin Z (Siemieniewicz *et al*., 2007; Kajla, 2005).

The chitosomal pathway of Chs3 is controlled by a multitude of regulatory proteins affecting Chs3 trafficking by protein interactions or posttranslational modifications (Lesage and Bussey, 2006). In the ER, Chs3 is palmitoylated by Pfa4 regulating the exit from the ER. Pfa 4 (protein fatty acyltransferase 4) is a member of the DHHC family of palmitoyltransferases. Ch3 is released from ER only upon interaction with the ER chaperone Chs7 and both processes may be linked, as Lam *et al*. (2006) could show that palmitoylation of Chs3 promotes the interaction of Chs7 and Chs3, while non-palmitoylated Chs3 only weakly interacts with Chs7; however, this could be an indirect effect. In addition, the mature form of Chs3 is highly glycosylated (Valdivia and Schekman, 2003), which in yeast is a dual step process occurring in the ER and in the Golgi network (Wilson *et al*., 2009). The exit from the trans-Golgi network (TGN) is controlled by the regulatory proteins Chs5 and Chs6 (Santos *et al*., 1997; Ziman *et al*., 1998).

Furthermore, Chs4 is needed for enzymatic activity of Chs3 and localization at the bud neck (Reyes *et al*., 2007; Trilla *et al*., 1997). At the bud neck Chs3 is linked to septins via Chs4 and Bni4 (DeMarini *et al*., 1997). Bni4 also recruits the catalytic subunit of protein phosphatase 1 (Glc7) to the bud neck in a temporal and spatial restricted manner, a process that assists in recruiting Chs3 by a yet unidentified substrate (Larson *et al*., 2008).

Chs3 is a phosphoprotein and its localization is controlled by phosphorylation. Valdivia and Schekman (Valdivia and Schekman, 2003) demonstrated that in the absence of the serine/threonine kinase Pkc1 the amount of phosphorylated Chs3 is significantly decreased. Moverover, Martinez-Rucobo *et al*., (2009) could identify twelve phosphorylation sites in Chs2, all located in the N-terminal regulatory domain. Four of them are presumably phoshorylated by the cyclin-dependent kinase Cdk1. Moreover, Lenardon *et al.* (2010) recently suggested that in *Candida albicans* CaChs3 is phosphorylated dependent on its only Cdk, Cdc28.

In insects, the existence of chitosomes is discussed controversially (Merzendorfer, 2006). Similar to the results reported in yeast, Cohen (1982) isolated chitosomes from crude extracts of *T. castaneum*. The isolated particles ranged from 50 nm to 250 nm in diameter. After addition of UDP-GlcNAc the

particals were associated with long microfibrils from about 10 nm to 80 nm. In larval midguts of *M. sexta* the chitin synthase is located at the apical tips of the microvilli of columnar cells. Immunostainings revealed that the proteins do not only localize to the plasma membrane but also to vesicle like structures inside the cytoplasm, which maybe chitosomes (Zimoch and Merzendorfer, 2002). It is still unclear, if *in vivo* the chitin synthases are already active in chitosomes or if they become activated after fusion of the vesicle with the plasma membrane. To visualize chitin in chitosomes, cross sections of *M. sexta* midguts were incubated with the purified chitin binding domain from a *M. sexta* chitinase (CBD) and subsequently chitin was stained via Cy3-linked CBD specific antibodies. By these efforts, chitin could not only be detected in the peritrophic matrix but also in the vesicle like structures inside the cytoplasm (Meissner; diploma thesis).

4.1.4 Posttranslational regulation: Regulatory subunits

The best characterized regulatory protein of Chs3 in yeast is Chs4, which is part of the active Chs3 complex. (Grabinska *et al*., 2007; Kozubowski *et al*., 2003; Trilla *et al*., 1997). In yeast, overexpression of Chs3 does not result in increased chitin levels, but overexpression of the Chs4 does (Ono *et al*., 2000). The exact mode of activation, by which Chs4 activates Chs3 is still unclear, however, this process requires an interaction between both proteins (Ono *et al*., 2000). Furthermore Chs4 has a dual role in Chs3 regulation. In addition to its function in the activation of Chs3, it is also involved in the recruitment of Chs3 to the bud neck (DeMarini *et al*., 1997; Trilla *et al*., 1997). DeMarini *et al*. (1997) further hypothesized that Bni4 functions as a linker protein between Chs4 and septins of the bud neck. However, further analysis of this process yielded a more complex picture involving also the catalytic subunit of a Type 1 serine/threonine protein phosphatase (Glc7), which is also necessary for Chs3 recruitment to the bud neck (Kozubowski *et al*., 2003; Larson *et al*., 2008). Chs4 carries a C-terminal CaaX motif and Grabinska *et al*. (2007) showed that Chs4 is indeed prenylated. The biological function of CaaX processing of Chs4 is discussed in the next chapter.

S. cerevisiae also contains the functional Chs4 homologue Shc1, which promotes chitin synthesis during sporulation but does not regulate chitin synthesis in vegetative cells (Sanz *et al*., 2002).

4.1.5 Posttranslational regulation: Proteolytic activation

Posttranslational regulation of chitin synthases by proteases was suggested already in the early 70's (Cabib and Farkas, 1971). Since then the zymogenic activation of chitin synthesis has been investigated extensively. The zymogenic character of chitin synthases was primarily deduced from two findings: firstly, chitin synthesis is stimulated by limited proteolysis, and secondly chitin synthase preparations contain polypeptides of lower molecular mass than theoretically expected, which are cleavage products of the full length chitin synthases (Au-Young and Robbins, 1990; Bartnicki-Garcia, 2006; Kang *et al*., 1984; Lending *et al*., 1991; Montgomery *et al*., 1984; Uchida *et al*., 1996).

In insects, trypsin-mediated stimulation of chitin synthesis has been reported, supporting a zymogenic nature of chitin synthases (Cohen and Casida, 1980; Mayer *et al*., 1980; Ward *et al*., 1991). Zimoch *et al*. (2005) demonstrated that the addition of trypsin stimulates chitin synthesis in crude extracts. Furthermore, Broehan *et al*. (2007) identified an interaction of a novel soluble chymotrypsin-like protease (MsCTLP1) with the extracellular C-terminal domain of MsChs2. MsCTLP1 is activated by trypsin cleavage, and activated chymotrypsin has been shown to stimulate chitin synthesis in crude midgut extracts as well.

In this thesis proteolytic activation was shown for the first time on the level of the purified chitin synthase complex. Chymotrypsin could activate the purified MsChs2 complex at least 3-fold, as could also trypsin, which was of particular interest as trypsin was not able to stimulate chitin synthase activity in 120,000 g membrane factions (Zimoch *et al*., 2005). The finding that the purified complex is activatable with trypsin or chymotrypsin might be explained by the assumption that solubilization of MsChs2 results in a conformational change of the protein so that an originally masked trypsin cleavage side gets accessible for proteolysis. Another explanation could be that trypsin-mediated proteolytic activation is inhibited in the 120,000 g membrane faction by an unknown

protein, which binds to MsChs2 and blocks the cleavage site. Solubilization of the MsChs2 complex would lead to the removal of the regulatory factor rendering it susceptible for proteolysis. Such a protein could be similar to yeast Chs4, since similar results were observed in *S. cerevisiae*. In yeast cell extracts Chs3 activity can not be activated by trypsin, which led initially to the hypothesis that Chs3 is non-zymogenic (Bulawa and Osmond, 1990). However, after treatment of cell extracts with detergents solubilizing Chs3, chitin synthase activity increased 10-fold upon incubation with trypsin (Choi *et al*., 1994). Trypsin incubation could also recover the activity of solubilized inactive Chs3 from *chs4*Δ mutant; *chs4*Δ however shows no structural similarities to known proteases nor hasany protease activity (Trilla *et al*., 1997). This leads to the assumption that proteolytic activation might be inhibited by Chs4 in non-solubilized Chs3. Solubilization of Chs3 would then lead to an disassociation of the activator Chs4 and accessible of the masked trypsin cleavage side.

Studies by Martinez-Rucobo *et al*. (2009) demonstrated that in case of Chs2 an unknown soluble serine or cysteine protease proteolytically activates Chs2 chitin synthesis. Still no protease has been identified in yeast that proteolytic actives chitin synthesis *in vivo*.

4.1.6 A novel role for Ste24 in posttranlational control of Chs3

A large scale screening done by Miller *et al*. 2005 showed a putative interaction between Chs3 and the protease Ste24. Yeast proteases are classified in 3 different groups (Jones, 1991). The first group includes vacuolar proteases; the second one contains cytosolic proteases and the third group contains membrane-bound proteases of the secretory pathway. The membrane bound metalloprotease Ste24 is a member of the third group; proteases of the secretory pathway are responsible for the activation and regulation of proteins, while vacuolar proteases are primarily involved in the degradation of proteins (Jones, 1991). The results presented in this thesis verified the interaction between Ste24 and Chs3 and clearly demonstrated that Ste24 modulates chitin synthesis in *S. cerevisiae*. But in which compartment does the interaction between Ste24 and Chs3 occur? Chs3 is synthesized at the rough ER and

transported via the Golgi network and trans-Golgi vesicles to the plasma membrane of the bud neck (Lesage and Bussey, 2006). Ste24 carries a C-terminal di-lysine motif (KKXX) and thus is retained in the ER (Tam *et al*., 2001). In addition Ste24 is also found at the inner nuclear membrane (Barrowman *et al*., 2008). Therefore, an interaction between Chs3 and Ste24 is only possible at the ER, because it is the only membrane compartment in which both proteins are, at least transiently, co-localized. The results presented in this thesis showed that mutated Ste24, which lacks proteolytic activity, does not modulate chitin levels; therefore Ste24 must influence chitin levels by cleaving a protein involved directly or indirectly in chitin synthesis.

The only known substrate of Ste24 in *S. cerevisiae* is the MF**a** precursor, which is cleaved by Ste24 at the C-terminal CaaX motif in a prenylation-dependent manner and at an undefined sequence at the N-terminus (Tam *et al*., 1998). In this thesis, it could be excluded that the Mf**a** cleavage is responsible for altered chitin levels in *ste24*Δ cells, since deletion of *ste24* leads not only to a decrease of chitin levels in *MAT**a*** cells but also in *MAT*α cells were the **a**-factor is not expressed. The sequence specificities for CaaX cleavage by Ste24 has been determined by site-directed mutagenesis (Trueblood *et al*., 2000). Genome analysis identified 35 yeast CaaX proteins that are potentially cleaved by Ste24 proteases at their C-termini (Trueblood *et al*., 2000). Since Chs3 does not possess a CaaX motif, it is presumably not a substrate for prenylation-dependent cleavage by Ste24. Moreover, western blot analysis performed in this thesis and by Cos *et al*. (1998) yielded no evidence for cleavage of Chs3 at the N-terminus. Therefore the question arises, how Ste24 modulates chitin synthesis in yeast. A plausible answer is that another protein involved in chitin synthesis might be a substrate for Ste24. Among the 34 proteins which possess a C-terminal CaaX motif recognized by Ste24, only one is directly linked to chitin synthesis and Chs3. This protein is Chs4, a known activator of Chs3 as described above (Trilla *et al*., 1997).

Proteins carrying a CaaX motif predicted to be cleaved by Ste24							
CHS4	YBL061C	CVIM	*RCY1*	YJL204C	CCIM	YJL118W	CCCS
CDC42	YLR229C	CAIL	*RFC5*	YBR087W	CCLD	YOL014W	CIIL
CST26	YBR042C	CFIF	*RHO1*	YPR165W	CVLL	YDL009C	CAVS
DAL3	YIR032C	CIII	*RHO2*	YNL090W	CIIL	YIR007W	CVIS
EDS1	YBR033W	CFFN	*RHO4*	YKR055W	CIIM	YGL082W	CVIM
EXG2	YDR261C	CASL	*RHO5*	YNL180C	CVIL	YDR307W	CLAK
GIS4	YML006C	CAIM	*SAM37*	YMR060C	CKYI	YJR128W	CMMI
LAS21	YJL062W	CALD	*SUA5*	YGL169W	CIQF	YPL191C	CVVM
MFA1	YDR461W	CVIA	*XDJ1*	YLR090W	CCIQ	YPR099C	CVST
MFA2	YNL145W	CVIA	*YDY1*	YNL064C	CASQ	YFL066C	CCVC
RAS1	YOR101W	CIIC	*YKT6*	YKL196C	CIIM	YGL069C	CVCC
RAS2	YNL098C	CIIS		YKL069W	CVFK		

Table 9. Potential Ste24 substrates in yeast – The standard gene names (if named), the systematic gene names of potential substrates and their corresponding C-terminal CaaX motif are listed in the table. Chs4 is highlighted in red. (modified after Trueblood *et al*., 2000)

But does Ste24 modulate chitin synthesis by cleaving Chs4? CaaX cleavage depends on prenylation, therefore the CFW phenotypes in wild-type, *ste24*Δ or *STE24*↑ of mutated Chs4^{C693S} cells were examined. While yeast cells expressing wild-type *CHS4* showed CFW resistance or hypersensitivity in response to deletion or overexpression of *STE24*, no observed effects were observed in cells expressing non-prenylated Chs4^{C693S}. The fact that the deletion or overexpression of *ste24* has no effect on chitin levels when Chs4 is non-prenylated, strongly suggests that Ste24 modulates chitin synthesis by cleaving prenylated Chs4.

Then however, the next question arises why Ste24 binds to Chs3 if it cleaves another substrate, namely Chs4? Soluble immature Chs4 is prenylated by the farnesyltransferase complex Ram1/Ram2 in the cytosol, whereas the following two CaaX processing steps take place at the ER membrane (Wright and Philips, 2006). It is still not clear how the prenylated CaaX proteins reach the sites of the

ER membrane, where they are further processed by Ste24 and Ste14. Prenylated Rho GTPases, which also have a CaaX motif, are found in the cytosol in a complex with RhoGDI. The chaperone RhoGDI binds the hydrophobic prenylated C-terminus of Rho in a binding pocket making the protein complex soluble (Hoffman *et al*., 2000). Wright and Philips (2006) suggested that the farnesyltransferase Ram1/Ram2 itself could act as a chaperone ensuring the transport to the ER membrane. After the attachment of isoprenoid lipid, the protein remains associated with the farnesyltransferase until it is bound by the CaaX-protease at the ER. Since no interactions between Chs4 and Ste24 were detected in yeast two-hybrid analyses, a model is proposed where Chs3 helps to recruit Chs4 and hence facilitates cleavage of its CaaX motif at the ER. Chs4 has been detected in the cytoplasm and at the plasma membrane accumulating at the bud neck during cytokinesis (Reyes *et al*., 2007). However, in this thesis it could be shown that Chs4 is also present in significant amounts at the ER (Figure 26). Perhaps Chs3, Chs4 and Ste24 are part of a transient complex formed at the ER that is required for Chs4 processing. This complex may also involve other ER proteins such as Chs7 and the methyltrasferase Ste14. At least the ER chaperone Chs7 binds to Chs3 and controls its export from the ER. Ste14 on the other hand is required for the last step of CaaX processing, *i.e.* carboxyl methylation (Romano *et al*., 1998; Trilla *et al*., 1999). Interestingly Miller *et al* (Miller *et al*., 2005) reported not only a putative interaction between Ste24 and Chs3, but also an interaction between Chs7 and Ste24, which may therefore facilitate stabilization of CaaX processing complex (Figure 29).

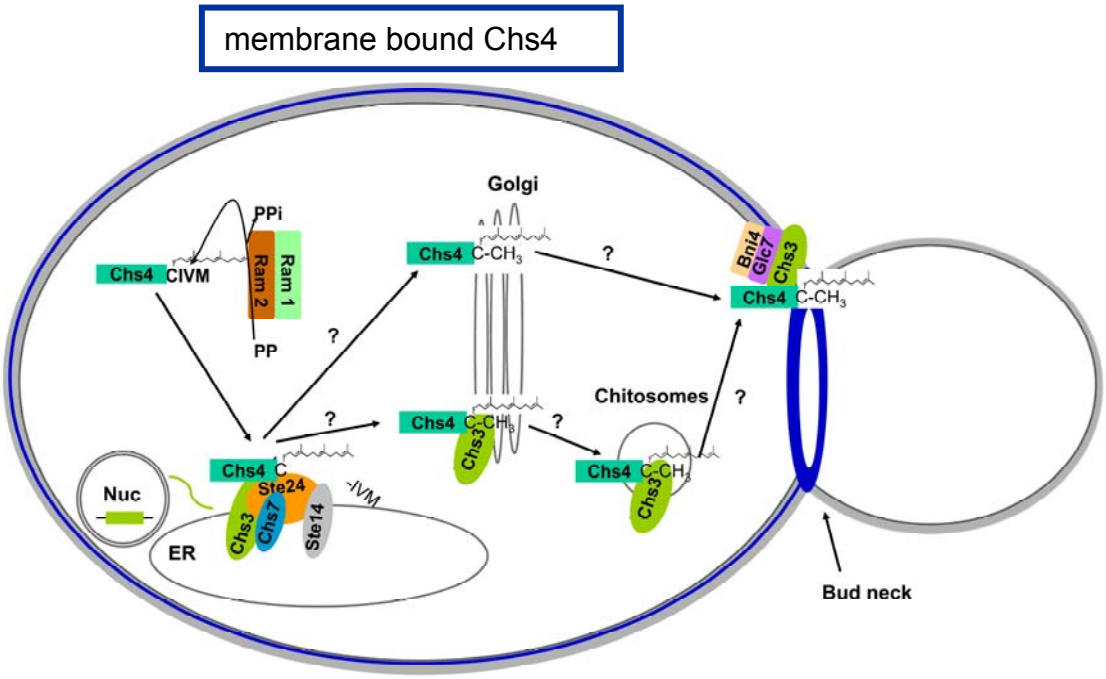


Figure 29. Putative model of a transient CaaX processing complex regulating Chs3 at the ER

The biological role of Chs4 prenylation has been analyzed previously with inconsistent results: while Grabinska *et al*. (2007) showed that yeast cells expressing a non-prenylated mutant of Chs4 exhibit 30% less chitin in their cell walls compared to wild-type cells, Reyes *et al*. (2007) claimed that the expression of non-prenylated Chs4 has only minor effects on the chitin content (-8%). In line with this result, Reyes *et al*. (2007) reported that the non-prenylated protein had only a minor effect on CFW resistance. However, Grabinska *et al*. (2007) describes a significant increase in CFW resistance in cells expressing a non-prenylated mutant of Chs4 compared to wild-type cells. Moreover Reyes *et al*. (2007) investigated the localization of non-prenylated Chs4 compared to wild-type Chs4 and showed that prenylation of Chs4 mediates membrane association. In contrast, Grabinska *et al*. (2007) analyzed the subcellular localization of non-prenylated and wild-type Chs4 by differential subcellular fractionation of yeast proteins and concluded that prenylation of Chs4 does not mediate plasma membrane localization nor membrane association of this protein. Taken together Reyes *et al*. (2007) state that Chs4 prenylation is required for its association with the plasma membrane but not for

its biological function to activate Chs3. Grabinska *et al*. (2007) on the other hand conclude the exact opposite: Prenylation is not required for plasma membrane localization, however it affects Chs3 activity. These contradictive results may be due to different genetic backgrounds and technical differences (Reyes *et al*., 2007).

The results presented in this thesis in parts support the data reported by Grabinska *et al*. (2007) and in other parts support the results by Reyes *et al*. (2007). The presented results showed that the CFW resistance is significantly increased in cells expressing non-prenylated Chs4 (Figure 24). Moreover the measured chitin levels in cells expressing non-prenylated Chs4^{C693S}, determined by two independent methods, are in accordance with those reported by Grabinska *et al*. (2007), demonstrating that the chitin content in these cells is decreased by about 30% to 35% (Figure 19).

On the other hand, this study demonstrates that prenylation of Chs4 is indeed necessary for membrane association, since non-prenylated Chs4 clearly localized to the cytosol (Figure 26), confirming the data by Reyes *et al*. (2007). Grabinska *et al*. (2007) investigated the subcellular localization of Chs4 in its prenylated and non-prenylated forms by fractionation. This method is rather indirect and harsh compared to GFP localization, since the yeast cells have to be lysated by detergents, which are known to interfere with protein interactions and cause artefacts.

	(Grabinska *et al*., 2007)	(Reyes *et al*., 2007)	this thesis (Meissner *et al*., 2010)
CFW resistance of non-prenylated Chs4 cells	+	(+)	+
Chitin Content in non-prenylated Chs4 cells	-30%	-8%	-34%
Prenylation mediates membrane association	-	+	+

Table. 10. CFW resistance, chitin content and membrane association of Chs4 as reported by Grabinska *et al.* (2007), Reyes *et al.*(2007) and this thesis (Meissner *et al.*, 2010)

Despite severe differences in localization, the chitin amount in yeast cells is only decreased by 30%. This raises the question why the prenylation has such a drastic effect on Chs4 localization and membrane association, while it has only a minor effect on its biological function in terms of Chs3 activation? Some CaaX-Proteins, like members of the Ras proteins, are not only prenylated but also palmitoylated (Hancock *et al*., 1989). The additional palmitoylation modification also increases the hydrophobicity and is responsible for protein targeting (Wright and Philips, 2006). However, so far there are no indications for palmitoylation of Chs4 in literature and a screening for potential palmitoylation sites (CSS-Palm2.0; www.csspalm.biocuckoo.org) did not reveal any apparent consensus sequence. Taking a closer look to the localization of Chs4 as shown in Figure 26, some non-prenylated Chs4 still localizes to the bud neck, which might explain the comparatively high chitin content in *chs4*^{C693S} cells. From this result it may be concluded that the interaction between Chs4, Chs3 and Bni4 is sufficient to recruit non-prenylated Chs4 partially to the bud neck where it can activate chitin synthesis (Figure 30).

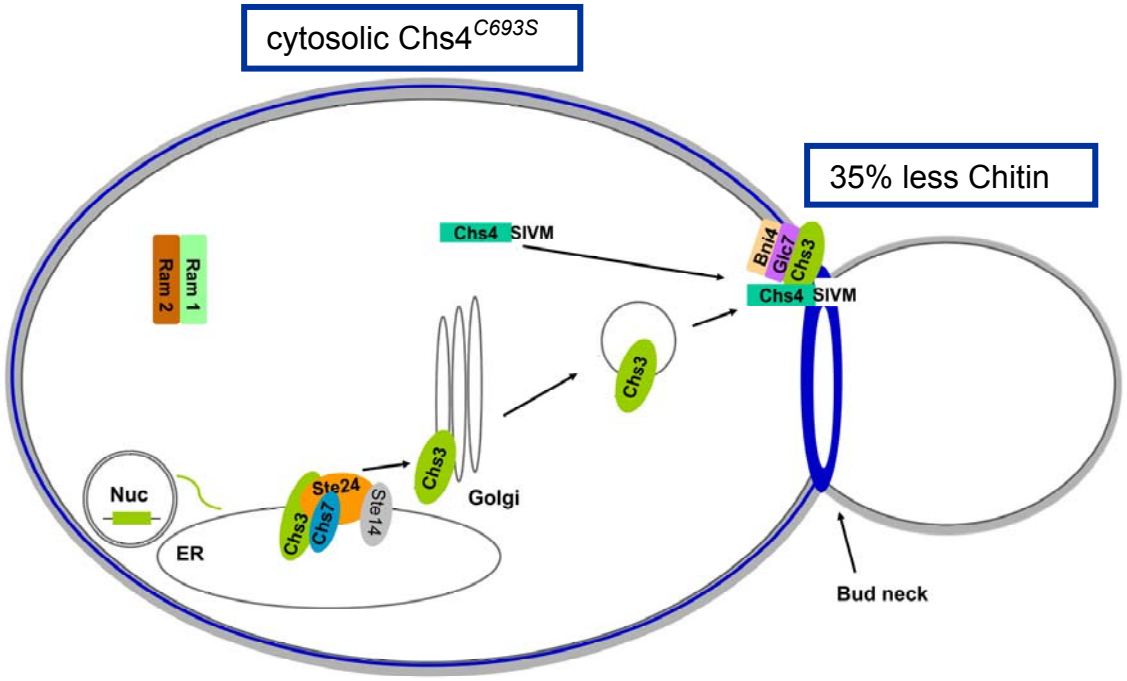


Figure 30. Putative model for Chs4^{C693S} transport

Does the prenylation depended cleavage of the CaaX motif of Chs4 also have an effect on Chitin synthesis? Like most studies Grabinska *et al*. (2007) and Reyes *et al*. (2007) investigated the role of CaaX processing in proteins by mutating the CaaX cysteine, rendering prenylation impossible. Because prenylation is a prerequisite for the activities of the CaaX protease and the methyltransferase, these studies are not informative regarding the role of the individual reactions that constitute CaaX processing. The result that *ste24* deletion mutants have significantly less chitin (-20%; Figure 19) points to an additional effect of the CaaX modification of Chs4 in chitin synthesis. Post-prenylation processing is of high physiological importance (Bergo *et al*., 2002) and in many cases the deletion of the CaaX protease or of the methyltransferase leads to mislocalization of the CaaX protein (Wright and Philips, 2006). Post sequential -aaX proteolysis and carboxyl methylation is required for membrane association of these proteins (Wright and Philips, 2006). In case of the 20 carbon geranylgeranyl modifications in Rho proteins, some findings suggested that geranylgeranyl modification itself confers sufficient hydrophobicity for membrane association in contrast to farnesylated CaaX proteins (15 carbon residues), whose hydrophobicity is just beneath the threshold for membrane association of the C-terminus of the protein. (Michaelson *et al*., 2005). Other results, however, suggest that geranylgeranyl modified Rho proteins also depend on post sequential -aaX proteolysis and carboxyl methylation (Papaharalambus *et al*., 2005). Interestingly, carboxyl methylation is reversible under physiologic conditions unlike prenylation and proteolysis (Chelsky *et al*., 1985). This may allow the cell to regulate the affinity of farnesylated proteins for membranes quite specifically (Wright and Philips, 2006).

Since Chs4 is not delocalized in *ste24*Δ cells or in the *ste24*Δ *rce1*Δ double mutants (Figure 26), cleavage of prenylated Chs4 or subsequent steps of CaaX processing carboxyl methylation appear not to be involved in membrane association of Chs4 (Figure 31). Nevertheless, deletion of *ste24* affects proper localization of Chs3-GFP at the bud neck (Figure 28) and decreases chitin levels (Figure 19). Therefore, the steps of CaaX processing of Chs4 subsequent to prenylation may contribute to the correct localization and activation of Chs3.

As the deletion of the methytransferase *ste14* has no influence on CFW-sensitivity (Figure 18), carboxyl methylation seems to play a minor role in this process. Furthermore, the results from the yeast two hybrid analysis suggest that prenylation is not necessary for the interaction between Chs3 and Chs4 (DeMarini *et al*., 1997); (Ono *et al*., 2000). However, an incompletely processed CaaX-side could still interfere partially with the interaction, leading to less Chs3 at the bud neck and ultimately less chitin. Nevertheless, deletion of ste24 moderately affects chitin synthesis and does not result in a complete loss of Chs3-GFP at the bud neck. Therefore, yeast cells may have mechanisms that partially compensate for the deficiency in Ste24 mediated CaaX cleavage, which may furthermore process additional substrates involved in chitin synthesis.

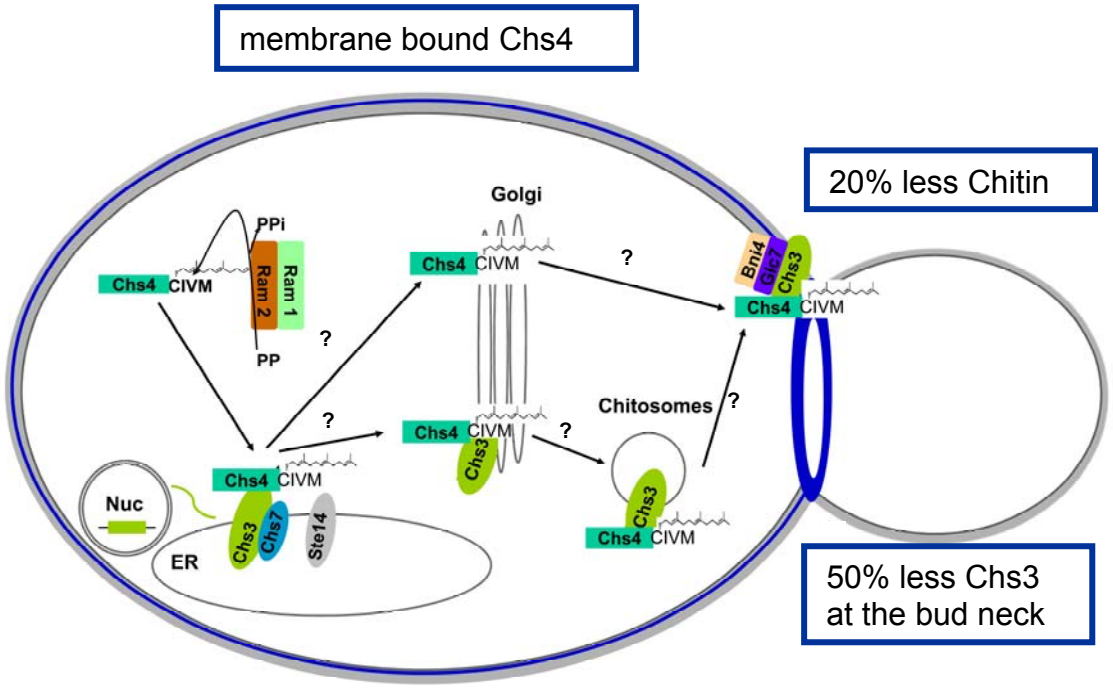


Figure 31. Putative model for Chs4 transport in *ste24*Δ cells.

4.1.7 Posttranslational regulation: Oligomerization

An interesting mechanism of posttranslational regulation of chitin synthases may be based on oligomerization of the mature chitin synthase complex, as shown by previous studies that demonstrated that a dissociation of yeast chitin

synthase complexes leads to a complete loss of activity (Ruiz-Herrera *et al*., 1980; Bartnicki-Garcia, 2006). Oligomerization of chitin synthase monomers may therefore be a universal property of these enzymes. The so far identified chitin synthase complexes (partially purified or purified) are all reported to be of high molecular masses (about 400–800 kDa) when analyzed under non-denaturing conditions (Bartnicki-Garcia, 2006; Kang *et al*., 1984; Lending *et al*., 1991; Montgomery *et al*., 1984; Ruiz-Herrera *et al*., 1980; Maue *et al*., 2009). If these complexes were analyzed under denaturing conditions, however, several polypeptides were identified with molecular masses being significant smaller than the predicted one of a chitin synthase monomer (Kang *et al*., 1984; Lending *et al*., 1991; Maue *et al*., 2009). Moreover, oligomerization has been reported for other glycosyltransferases. Cellulose synthase form hexagonal rosette complexes. Six monomers or three dimers form an oligomeric subunit of which six form the rosette super complex (Richmond, 2000; Somerville, 2006). Not only the closely related cellulose synthases but also the more distantly related glucosylceramide synthases and glycogen synthases form high molecular mass complexes (Doblin *et al*., 2002; Horcajada *et al*., 2006; Somerville, 2006). The oligomerization of transmembrane glycosyltransferases may facilitate the formation of pores in the membrane through which the nascent polymers are translocated (Cohen, 2001).

The findings of this thesis are in line with previously reported characterizations of chitin synthases. With a molecular weight of about 520 kDa, the chitin synthase complex from the *M. sexta* midgut is a typical member of this protein family. Electron microscopy and negative staining revealed molecules of 10 nm in diameter, a size which fits nicely with the observed molecular mass. The observed dimer of trimer and trimer of trimer in Figure 10 might be fragments of an even larger super complex similar to cellulose synthase rosette complex (Richmond, 2000).

The yeast two hybrid analysis presented in this thesis verified that a soluble domain of Chs3 can interact with itself, presumably mediating oligomerization. In addition, a coiled-coil region at the C-terminus, which is predicted to face the extracellular site, may be involved in protein–protein interaction, vesicle fusion or oligomerization (Merzendorfer and Zimoch, 2003; Skehel and Wiley, 1998).

Whether oligomerization is important for enzyme activity, or chitin translocation *in vivo* remains to be determined.

4.2 Yeast two hybrid analyses and topology of Chs3

Protein interaction between Chs3 and Chs4 was identified previously by classical yeast two-hybrid analysis (Ono *et al*., 2000), while an interaction between Chs3 and Ste24 was detected in a large-scale split ubiquitin screening (Miller *et al*., 2005). DeMarini *et al*. (1997) furthermore demonstrated that a 700 amino acid domain comprising the N-terminal region of Chs3 mediates binding to Chs4 and, moreover, homo-dimerization of Chs3. In this thesis, the soluble domains of Chs3 (C1, C3, C4, C7), Ste24 (S2, S6, S8) and the soluble protein Chs4 were tested in a classical yeast two hybrid analysis for mutual interaction. Chs4, Ste24 and Chs3 itself were identified as interaction partners of Chs3.

In addition, the binding site of Chs3 was narrowed down. Intriguingly only one domain, the C3 domain, is responsible for all three interactions. This domain, representing the region between amino acid positions 226 and 452, interacts with a so far unidentified region of Chs4, the Ste24 domains S2, S8 and with itself. However, this domain showed no interaction with C1, C4, C7 and S6 domains, supporting the specificity of the observed interactions.

In silico predictions of the Chs3 topology yielded inconclusive results with 6-9 postulated transmembrane helices and a C3 domain that is exposed either to the cytoplasm or to the extracellular space. The two-hybrid results presented in this thesis strongly suggest that the C3 domain is located intracellular, since an interaction between Chs4, which is associated with the membrane at the cytoplasmic side, and the C3 domain of Chs3 can only take place in the same compartment. Furthermore the C3 domain interacts with a cytoplasmic interface of Ste24 formed by the S2 and S8 domains (Tam *et al*., 2001). Additional hints also suggest a cytoplasmic localization of C3: Lam *et al*. (2006) demonstrated that Chs3 is palmitoylated by Pfa4, whose catalytic domain faces the cytoplasm, and that this posttranslational modification is one prerequisite for its exit from the ER. Screenings for palmitoylation sites with CSS-Palm2.0 identified only two putative type III motifs in Chs3, both located at the edge of the C3 domain

(amino acid positions 227-231, 446-451; Ren *et al*., 2008) additionally supporting its cytosolic location. In a more recent publication, Barfield *et al*. (2009) identified a plasma membrane signal (IXTPK) sequence in the cytosolic tail of the cell fusion protein Fus1 (Figure 32). Sequence analysis identified this cytosolic signal also within the C3 domain of Chs3. The catalytic domain C4, which follows the C3 domain, is also cytosolic. This was shown by PhoA fusion studies performed with the rhizobial chitin synthase NodC, which revealed that the catalytic domain of this β-glycosyltransferase is exposed to the cytoplasm (Barny *et al*., 1996).

The C-terminal C7 domain of yeast Chs3 exhibits coiled-coil regions like insect Chs1 enzymes, which may be involved in protein–protein interactions, vesicle fusion or oligomerization (Merzendorfer and Zimoch, 2003; Skehel and Wiley, 1998). These coiled-coil regions are commonly found at the extracellular site of the membrane (Skehel and Wiley, 1998). Therefore, a topology model is proposed in which the C3 domain precedes the catalytic domain, both facing the cytoplasm and an extracellular C7 domain (Figure 13B; Figure 16; Figure 32). Perhaps the predicted transmembrane helices between C3 and the catalytic domain C4 just attach to the membrane, possibly anchored by a palmitoyl residue.

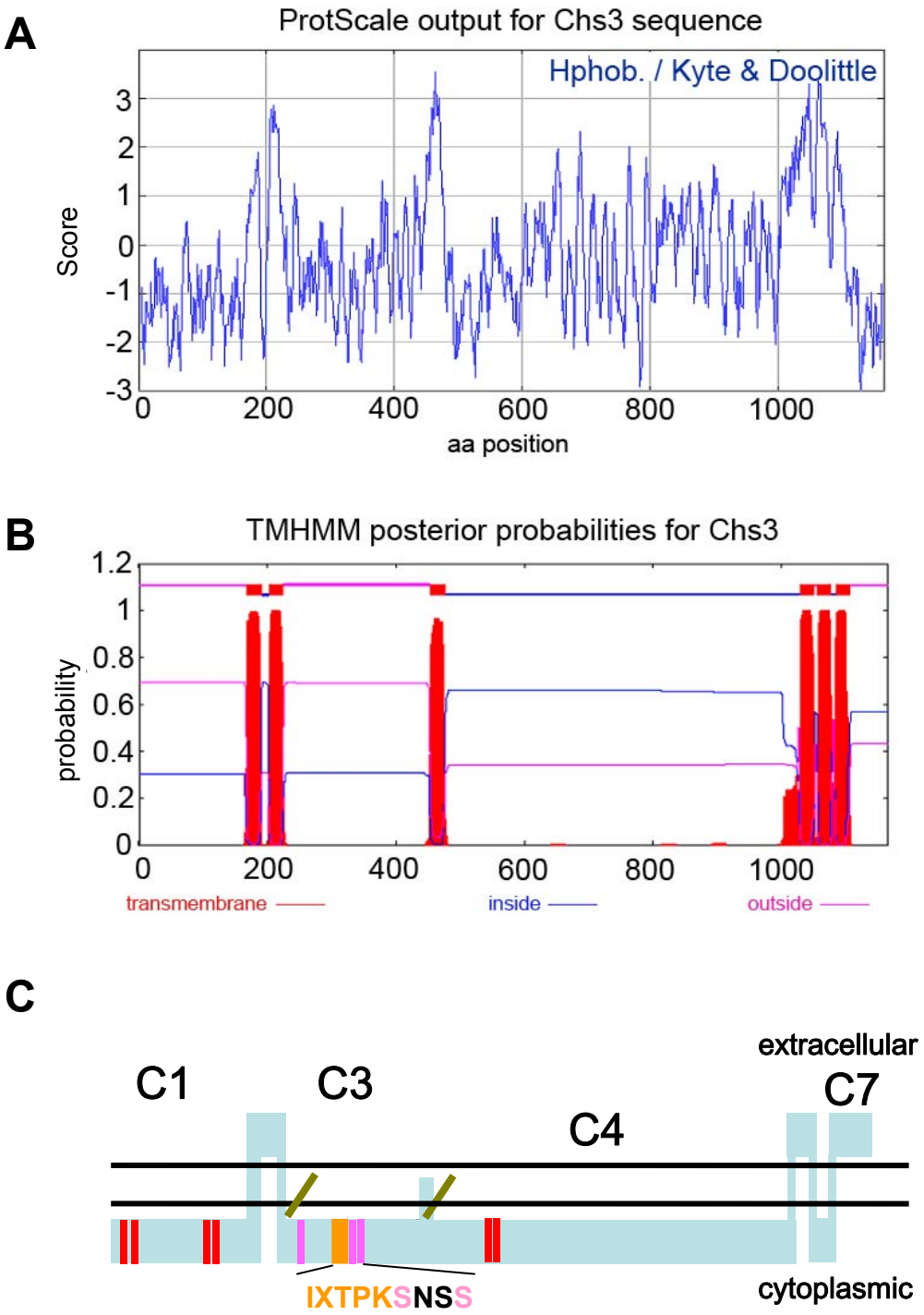


Figure 32. Putative topology of Chs3 – (**A**) Hydropathy plot of Chs3. The plot was calculated with ProtScale (ExPASy Proteomics Server). (**B**) Prediction of transmembrane helices in Chs3 (TMHMM Server v. 2.0). (**C**)Putative membrane structure of Chs3 (orange: cytosolic plasma membrane signal IXTPK; red: identified phosphorylation sites S29, S32, S147, T148, S537 and T538; pink putative phosphorylation sites at position S229, S302 and S305; green: putative palmityl residue)

4.3 The C3-domain of Chs3 is a multiple interaction and localization domain

An important question that arises upon analyzing the data from this study is the point, how yeast cells regulate and coordinate the various Chs3 interactions *in vivo*? The results of the Y2H analysis give first clues about how the various protein interactions of Chs3 are regulated.

As described above, Chs3 is transported by the chitosomal pathway, coordinated by a number of protein interactions. But how is Chs3 activation and transport regulated?

In a working model, this control of Chs3 activity and/or transport could be mediated by the C3 regulatory domain, which may coordinate specific protein interactions in a spatial-temporal manner. Chs3 is synthesized at the rough ER, where it is retained until it is correctly folded in a Chs7-dependent way and processed by palmitoylation; during its transient stay at the ER Chs3 appears to interact via its C3 domain with Chs4 and Ste24 to form a transient complex that is required for Chs4 processing. In the TGN membrane, the C3 domain (signal sequence IXTPK) interacts through Chs5 with the exomer complex responsible for the transport from the Golgi to the plasma membrane (Barfield *et al*., 2009). Studies by Valdivia and Schekman (2003) demonstrated that this transport is also regulated by phosphorylation, mediated by the serine/threonine kinase Pkc1. Several phosphorylation sites were identified in Chs3 by High Throughput screening (S29, S32, S147/T148, S537, and T538; PhosphoGRID and PhosphoPep Database). All identified site were either found in the N-terminal C1 or catalytic domain C4. Since only a few peptides from Chs3 were identified in the screenings, additional phosphorylation sites might exist. Phosphorylation predictions in yeast revealed three putative phosphorylation sites in the C3 domain (S229, S302 and S305; NetPhosYeast 1.0 Server). After passing the chitosomes, the C3 domain interacts with Chs4 as a prerequisite for localization in the plasma membrane at the bud neck. Finally, C3 interacts with itself leading to enzymatically active Chs3 oligomers. Further studies have to be carried out to determine the correct topology of the regulatory C3 and its specific regulatory function in Chs3 transport.

5 Summary

In this thesis the regulation of chitin synthases by proteolytic activation has been analyzed in yeast and insects. It was shown that the solubilized chitin synthase 2 of *Manduca sexta* (MsChs2) is an oligomeric complex of about 10 nm in diameter. In contrast to MsChs2 in membrane fractions, it can be activated by trypsin and chymotrypsin in the solubilized and purified state.

In yeast, proteolytic activation of chitin synthases has been described almost 40 years ago. However, no protease has been identified stimulating chitin synthesis *in vivo*. Recently, Martinez-Rucobo *et al*. (2009) demonstrated, that the chitin synthase 2 (Chs2) of *Saccharomyces cerevisiae* is activated by a still unknown soluble endogenous protease. A global screening for protein-protein interactions indicated that the metalloprotease Ste24 interacts with chitin synthase 3 (Chs3). Ste24 is a membrane-integral CaaX protease residing in the endoplasmic reticulum (ER). In yeast, the only known substrate of Ste24 is the mating factor **a** (MF**a**) precursor. The interaction between Ste24 and Chs3 was verified by yeast two hybrid analysis and the interacting domains were mapped. Further investigations focused on the characterization of Ste24's influence on chitin synthesis. Growth tests demonstrated that *ste24*Δ mutants are resistant to Calcofluor White (CFW). Mutant cells expressing a catalytically inactive version of Ste24 were also CFW resistant and showed a decrease in chitin levels. Overexpression of *STE24* resulted in hypersensitivity to CFW and a slight increase in chitin levels. The CFW phenotype of *ste24*Δ cells could be rescued by its human and insect orthologues. Additionally, Chs3-GFP localized less frequently at the bud neck in *ste24*Δ cells. Although Chs3 binds to Ste24, it appears not to be a substrate of this protease. Instead Ste24 modulates the chitin synthesis by cleaving the CaaX motif of prenylated Chs4, a known activator of Chs3, since in cells expressing non-prenylated Chs4, deletion or overexpression of Ste24 had no influence on chitin synthesis. Moreover, the data suggests that Chs3 and Ste24 form a complex in the ER that facilitates proteolytic activation of Chs4, a known activator of Chs3 with a C-terminal CaaX motif, leading to a more efficient localization of Chs3 at the plasma membrane.

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7 Appendix

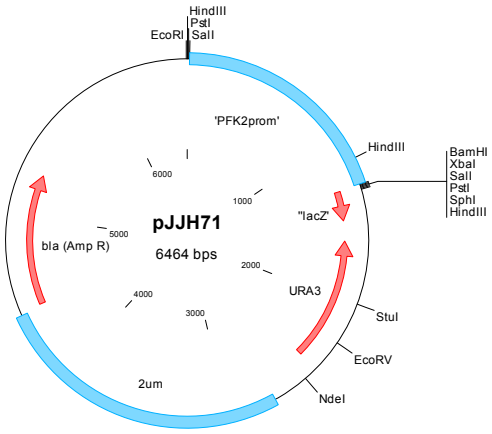
7.1 Abbreviations

aa	Amino acid
BSA	Bovine serum albumin
cDNA	complementary Deoxyribonucleic acid
CFW	Calcofluor White
chs	Chitin synthase
CTLP	Chymotrypsin-like peptidase
Da	Dalton
DNA	Deoxyribonucleic acid
EDTA	ethylenediaminetetraacetic acid
ER	Endoplasmic reticulum
f.c.	final concentration
GFP	Green fluorescent protein
IgG	Immunoglobulin G
IPTG	Isopropyl β-D-1-thiogalactopyranoside
MAT	Mating type
MF**a**	Mating factor a
MOPS	3-(N-morpholino)propanesulfonic acid
mRNA	Messenger RNA
Ms	*Manduca sexta*
ORF	Open reading frame
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
pH	negative logarithm (log_{10}) of the hydroxonium concentration
RNA	Ribonucleic acid
rpm	Rounds per minute
Sc	*Saccharomyces cerevisiae*
SC	Synthetic complete medium
ste	Sterile

TBS	TRIS buffered saline
Tc	*Tribolium castaneum*
TRIS	tris(hydroxymethyl)aminomethane
U	Unit
UV	Ultraviolet radiation
v/v	volume/volume percentage
w/v	weight/volume percentage
x g	-times gravitational acceleration
X-Gal	bromo-chloro-indolyl-galactopyranoside
YPD	Yeast extract peptone dextrose (rich medium)
ZmpSte24	zinc metalloprotease Ste24 homolog

Nucleotides and amino acids are represented with the single letter code (IUPAC-IUB Commission on Biochemical Nomenclature).

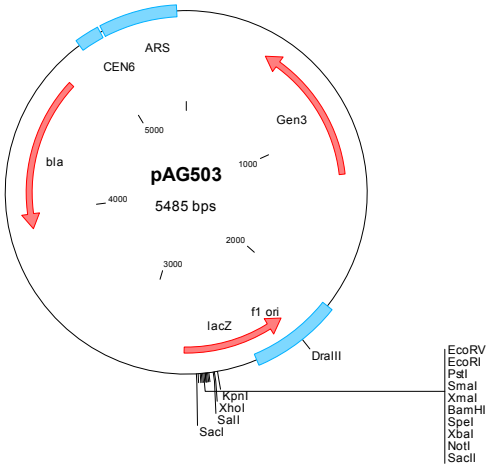
7.2 Plasmids



pJJH71 sequence features

Type	Start	End	Name
REGION	13	1290	PFK2 promotor
GENE	1297	1488	lacZ MCS
GENE	2423	1620 C	URA3
REGION	4410	2710 C	2µ ori
GENE	4428	5282	bla (Amp R)

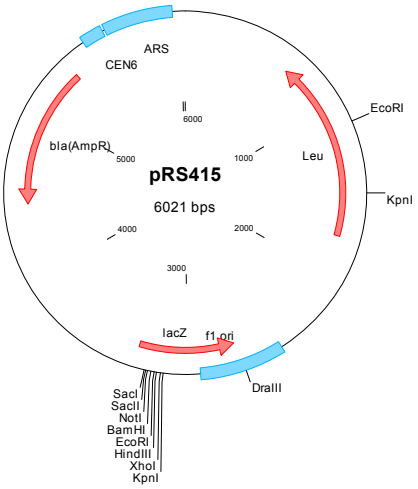
Figure 33. The pJJH71 vector map and sequence features



pAG503 sequence features

Type	Start	End	Name
GENE	1271	462 C	Gen3 G418 resistance
REGION	2393	1961 C	f1 origin of replication
GENE	2754	2176 C	lacZ MCS
GENE	4771	3911 C	bla (Amp R)
REGION	4935	5053	CEN6 (yeast centromere)
REGION	5066	5439	ARS Histone H4

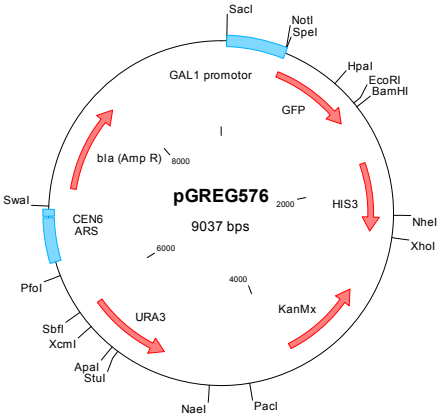
Figure 34. The pAG503 vector map and sequence features



pRS415 sequence features

Type	Start	End	Name
GENE	1769	663 C	Leu2 CDS
REGION	2929	2474 C	f1 origin of replication
GENE	3290	2712 C	lacZ
GENE	5307	4447 C	bla (AmpR)
REGION	5448	5566 C	CEN6 (yeast centromere)
REGION	5579	5952	ARS Histone H4

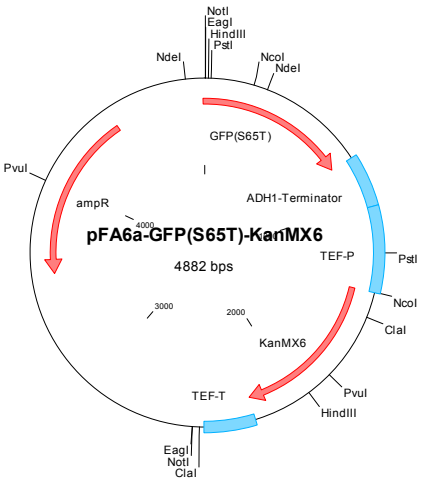
Figure 35. The pRS415 vector map and sequence features



pGREG576 sequence features

Type	Start	End	Name
REGION	47	549	GAL1 promotor
GENE	550	1341	GFP
GENE	1773	2432	HIS3
GENE	3833	3024 C	KanMx
GENE	5880	5077 C	URA3
REGION	6366	6739	ARS Histone H4
REGION	6752	6810	CEN6 yeast centromere
GENE	7011	7871	bla (Amp R)

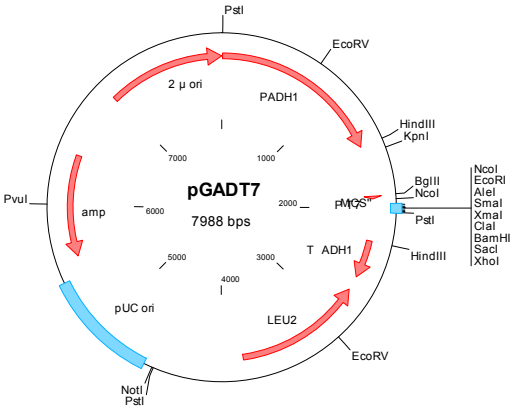
Figure 36. The pGREG576 vector map and sequence features



pFA6a-GFP(S65T)-KanMX6 sequence features

Type	Start	End	Name
REGION	775	1012	ADH1-Terminator
REGION	1012	1401	TEF-Promotor
GENE	1401	2210	KanMX
REGION	2211	2444	TEF-Terminator
GENE	4413	3553 C	bla (Amp R)
GENE	4874	774	GFP(S65T)

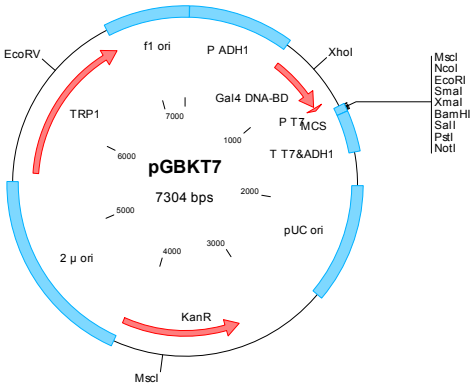
Figure 37. The pFA6a-GFP(S65T)-KanMX6 vector map and sequence features



pGADT7 sequence features

Type	Start	End	Name
GENE	7	1479	ADH1 promoter
GENE	1905	1927	T7 promoter
GENE	1942	1968	HA epitope tag
REGION	1969	2041	MCS
GENE	2280	2605	ADH1 terminator
GENE	3814	2723 C	LEU2
REGION	4581	5418	pUC ori
GENE	6432	5575 C	bla (Amp R)
GENE	6998	7988	2µ ori

Figure 38. The pGADT7 vector map and sequence features



pGBKT7 sequence features

Type	Start	End	Name
REGION	30	736	ADH1 promoter
GENE	762	1202	Gal4 DNA-BD
GENE	1212	1235	T7 promoter
REGION	1248	1280	c-Myc
REGION	1281	1334	MCS
REGION	1335	1610	T7&ADH1
REGION	1838	2636	pUC ori
GENE	4144	3222 C	KanR
REGION	4148	5493	2µ ori
GENE	5559	6755	TRP1
REGION	6756	29	f1 ori

Figure 39. The pGBKT7 vector map and sequence features

7.3 Curriculum Vitae

Personal Data

Name:	Derek Gilbert Meissner
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	derek.meissner@gmx.de
Day of birth:	04/24/1976
Birthplace:	San Nicolas, Argentina
Marital status:	unmarried

Studies and education

2005-2010	PhD student at the University of Osnabrück supported by the SFB 431 Associated member of the Graduate-College 612 Thesis title: “Proteolytic activation of chitin synthesis”
1998-2004	Studies in biology at the University of Osnabrück, Diploma in biology Thesis title: “Untersuchung der Chitinbindedomäne der Chitinase von *Manduca sexta*“
1989-1996	Weser Gymnasium Vlotho, Abitur (High school graduation)

7.4 Publications

Meissner, D., Odman-Naresh, J., Vogelpohl, I. and Merzendorfer, H. (2010) A Novel Role of the Yeast CaaX Protease Ste24 in Chitin Synthesis. Mol. Biol. Cell.2010; **21**: 2425-2433. Epub ahead of print 2010 May 26

Maue, L., Meissner, D. and Merzendorfer, H. (2009) Purification of an active, oligomeric chitin synthase complex from the midgut of the tobacco hornworm. Insect Biochem Mol Biol. 2009 Sep; **39**(9):654-9. Epub 2009 Jul 2.

7.5 Erklärung über die Eigenständigkeit der erbrachten wissenschaftlichen Leistung

Ich erkläre hiermit, dass ich die vorliegende Arbeit ohne unzulässige Hilfe Dritter und ohne Benutzung anderer als der angegebenen Hilfsmittel angefertigt habe. Die aus anderen Quellen direkt oder indirekt übernommenen Daten und Konzepte sind unter Angabe der Quelle gekennzeichnet.

Bei der Auswahl und Auswertung folgenden Materials haben mir die nachstehend aufgeführten Personen in der jeweils beschriebenen Weise entgeltlich / unentgeltlich geholfen.

1. Im Rahmen von Diplomarbeiten wurde ein Teil der Laborarbeiten gemeinsam mit Studenten durchgeführt. Dabei ist die Abbildung 17 in Zusammenarbeit mit Jothini Odman-Naresh und die Abbildung 20A in Zusammenarbeit mit Inga Vogelpohl entstanden.

2. Die elektronenmikroskopischen Aufnahmen vom MsChs2 Komplex (Abbildung 10) wurde von Anke C. Terwisscha van Scheltinga am Max Planck Institut für Biophysics, Department of Structural Biology in Frankfurt aufgenommen.

Weitere Personen waren an der inhaltlichen materiellen Erstellung der vorliegenden Arbeit nicht beteiligt. Insbesondere habe ich hierfür nicht die entgeltliche Hilfe von Vermittlungs- bzw. Beratungsdiensten (Promotionsberater oder andere Personen) in Anspruch genommen. Niemand hat von mir unmittelbar oder mittelbar geldwerte Leistungen für Arbeiten erhalten, die im Zusammenhang mit dem Inhalt der vorgelegten Dissertation stehen.

Die Arbeit wurde bisher weder im In- noch im Ausland in gleicher oder ähnlicher Form einer anderen Prüfungsbehörde vorgelegt.

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(Ort. Datum) (Unterschrift)

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SUPER VIELEN DANK AN ALLE