Crosslinks in the cell wall of budding yeast control morphogenesis at the mother–bud neck

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Summary

Previous work has shown that, in *cla4Δ* cells of budding yeast, where septin ring organization is compromised, the chitin ring at the mother–daughter neck becomes essential for prevention of neck widening and for cytokinesis. Here, we show that it is not the chitin ring *per se*, but its linkage to β(1-3)glucan that is required for control of neck growth. When in a *cla4Δ* background, *crh1Δ crh2Δ* mutants, in which the chitin ring is not connected to β(1-3)glucan, grew very slowly and showed wide and growing necks, elongated buds and swollen cells with large vacuoles. A similar behavior was elicited by inhibition of the Crh proteins. This aberrant morphology matched that of *cla4Δ chs3Δ* cells, which have no chitin at the neck. Thus, this is a clear case in which a specific chemical bond between two substances, chitin and glucan, is essential for the control of morphogenesis. This defines a new paradigm, in which chemistry regulates growth.

Key words: Morphogenesis, Yeast, Cell wall, Chitin, Glucan

Introduction

Morphogenesis is an essential process during development in all organisms. It must be under strict control to produce precise shapes and reproduce them indefinitely. Despite its importance, little is known about its regulation at the molecular level. For many years, we have studied the structure and synthesis of the yeast cell wall, which determines cell shape, and of a specialized portion of the wall, the septum, and used them as models for morphogenesis (Cabib et al., 2001). We have especially focused on the synthesis and function of chitin, a minor component of the cell wall that is essential for cell survival. A chitin ring is formed at bud emergence at the neck between mother and daughter cell. Synthesis of this chitin is catalyzed by chitin synthase 3 (Chs3). At cytokinesis, chitin synthesis starts again, in the form of a primary septum that advances centripetally to close the channel between mother and daughter cell, in synchrony with the contraction of an actomyosin ring (Lippincott and Li, 1998; Schmidt et al., 2002). Here, the polysaccharide synthesis is catalyzed by chitin synthase 2 (Chs2). After the primary septum is completed and secondary septa begin to be formed, chitin is also laid down in the cell wall of the daughter cell, under Chs3 control (Shaw et al., 1991).

It should be noted that during the cell cycle the diameter of the mother–bud neck, where the chitin ring and the primary septum arise, remains the same throughout. Therefore, some mechanism must limit growth to the bud and block it at the boundary between mother and daughter cell. The importance of this growth control is obvious, because in *Saccharomyces cerevisiae*, in contrast with fission yeast or animal cells, the site for cytokinesis is created and partially organized when and where the bud emerges. For the tightly orchestrated process of cytokinesis to take place smoothly, it would seem imperative to maintain a strict control on the topography of that site.

The function of Chs2 and the primary septum are clear, but that of the chitin ring was not, especially because it could be abolished by mutation of the *CHS3* gene (Shaw et al., 1991) without affecting budding or cell survival. In the past, we ran a genetic screen for mutants synthetically lethal with *chs3* (Schmidt et al., 2003). Two genes emerging from this screen were *CLA4*, a protein kinase, and *CDC11*, a septin gene. Cla4 shares some essential function with Ste20, but has also some functions of its own, one of which is its participation in the assembly of the septin ring, a structure required for cytokinesis (Longtine and Bi, 2003; Versele and Thorner, 2004). In *cla4* mutants, the septin ring is still formed, but it is often partially defective (Cvrcková et al., 1995; Schmidt et al., 2003).

When *cla4Δ* unbudded cells were allowed to bud in the presence of nikkomycin Z, an inhibitor of Chs3, the chitin ring did not form and elongated buds with very wide necks were generated, followed by growth arrest and lysis (Schmidt et al., 2003). A similar course of events took place in cells containing the *cdc11* mutation isolated in the same screen (Schmidt et al., 2003). These results show that the chitin ring becomes essential for maintenance of the neck structure when the septin ring is compromised and suggests that septins and chitin ring share a redundant function in the control of growth at the neck. Thus the chitin and the septin ring would control growth at the neck by different mechanisms.

The septin ring is made out of proteins and is positioned internally to the plasma membrane, therefore it is unlikely that it would act in a mechanical fashion. However, it has been found in several cases to act as a barrier to the movement of proteins in the plasma membrane (Caudron and Barral, 2009; Orlando et al., 2011). This seems a probable way in which it would act here, preventing proteins needed for cell wall synthesis from reaching the neck.

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Although the existence of the chitin ring had been known since the early 1970s (Hayashibe and Katohda, 1973; Cabib and Bowers, 1975), this was the first time that a function could be assigned to it. How could the effect of the ring be explained? One possibility was that it was working in a mechanical fashion, by making the wall more rigid at the neck. However, in *chs3* mutants (Shaw et al., 1991) or in wild-type cells treated with Nikkomycin Z, which lack the chitin ring, no widening of the neck is observed. Therefore, the cell wall appears to be able to maintain the shape of the neck in the absence of the chitin ring, so long as the septin ring is normal. Thus, we were lead to seek a different interpretation, based on the cell wall structure that we previously elucidated (Kollár et al., 1997). In this structure, chitin is partially attached to the nonreducing end of β(1-3)glucan and partially to a β(1-3)-linked glucose side chain of β(1-6)glucan, whereas β(1-6)glucan, to which the mannoproteins are linked, is also bound to nonreducing ends of β(1-3)glucan. We reasoned that at the neck, where the concentration of chitin is high, it might be mostly linked to β(1-3)glucan. Thus, it would compete with the attachment to the same sites of β(1-6)glucan, which is linked to the cell wall after β(1-3)glucan is synthesized (Roh et al., 2002a). As a consequence, mannoprotein also could not become a part of the cell wall (Schmidt et al., 2003). The linkage of chitin to end residues of β(1-3)glucan might also interfere with the metabolism of this polysaccharide, the main structural component of the cell wall. Thus, synthesis of the cell wall at the neck would eventually be stopped. As previously discussed (Cabib et al., 2012), the growth control systems would be switched on after a cell wall of normal composition was laid down at the neck.

In so far as the chitin ring is concerned, this hypothesis makes three predictions. First, that in the neck region chitin should be mostly attached to β(1-3)glucan, whereas it might be preponderantly bound to β(1-6)glucan in lateral walls. Second, that the structure of β(1-3)glucan at the neck should be somehow different from that of lateral walls, to reflect a less active metabolism. Third, that the mere presence of chitin would not block growth at the neck, unless the chitin is glucan-bound.

To verify the first prediction, we devised new procedures for the quantitative analysis of chitin crosslinks with other polysaccharides (Cabib and Durán, 2005). By applying this methodology to a mutant blocked at cytokinesis at a nonpermissive temperature, we found that the linkage of chitin to β(1-3)glucan was preponderant at the neck, whereas that to β(1-6)glucan predominated in lateral walls (Cabib and Durán, 2005), in accordance with expectations.

For the second prediction, methodologies for the isolation and fractionation of both free β(1-3)glucan and β(1-3)glucan attached to chitin were devised. The results showed that the bulk β(1-3)glucan consisted of two fractions, one soluble in alkali and highly polydisperse, the other insoluble and of very large molecular weight (Cabib et al., 2012). The large-size crosslinked polysaccharide was identified as part of the cell wall structural network, whereas the polydisperse material corresponded to the fraction being remodeled during cell growth (Cabib et al., 2012). The β(1-3)glucan attached to chitin, which is restricted to the neck, only consisted of high molecular weight material, supporting the idea that the cell wall at the neck is quiescent (Cabib et al., 2012).

Although these results were in agreement with our expectations, stronger evidence was needed to prove our third and crucial prediction, the subject of the present study; that is, that it is the binding of chitin to glucan, rather than its mere presence, that controls growth at the neck. To obtain this evidence, it was necessary first to uncover the mechanism by which the crosslinks between the polysaccharides are generated. Because this process takes place in the periplasmic space, where no ATP or other energy source is available, it was earlier suggested that the crosslinking would occur by transglycosylation, a neutral reaction in terms of free energy change (Cabib et al., 1988). This turned out to be the case. We showed that Crh1 and Crh2 act as transglycosylases to transfer chitin chains to β(1-6)glucan (Cabib et al., 2007) and also to β(1-3)glucan (Cabib, 2009). Armed with this knowledge, we could test the third prediction of our proposal, by deleting *CRH1* and *CRH2* in a *cla4Δ* mutant, thus eliminating chitin–glucan linkages without presumably affecting chitin content. As will be shown below, the results confirmed our hypothesis.

Results

Similar morphologies in *cla4Δ chs3Δ* and *cla4Δ crh1Δ crh2Δ* cells

Despite the synthetic lethality between *chs3* and *cla4* mutants observed in a genetic screen, we were able to isolate a *cla4Δ chs3Δ* strain (YMS90, see supplementary material Table S3) (Schmidt et al., 2003) in a YPH499 background. In an attempt to understand the reason why this double mutant survived, we mated it to a wild type with the same genetic background as YPH499 but the opposite mating type, and carried out tetrad analysis of the diploid. When mutations in two genes segregate, one would expect an equal number of spores with each one of the mutations, with no mutations and with both mutations. As shown in supplementary material Table S1, the recovery of the double mutant was one order of magnitude less than that of the single mutants or wild type. This might indicate that survival of the double mutant is the result of multiple factors, such as suppressor mutations arising during the generation of the double mutant strain. Another, more likely possibility, is that survival was initially resulting from the different genetic backgrounds of YPH499 and ECY101, the strain with which the genetic screen was carried out (Schmidt et al., 2003), but germination of double mutant spores is severely impaired. This would not be surprising, in view of the many defects of the double mutant. This strain grew very slowly and exhibited a conspicuously aberrant morphology, with extremely elongated buds, wide necks and bloated cells with large vacuoles (Fig. 1B, compare with Fig. 1A). Because of the lack of Chs3, the only chitin in these cells is that of the primary septum and little staining is visible with Calcofluor White (CFW) (Fig. 1B). To obtain a strain that would still have all the chitin but would be unable to link it to glucans, we deleted *CRH1* and *CRH2* in a *cla4Δ* mutant. The resulting triple mutant, NBT017, was viable, but showed slow growth and temperature sensitivity that was suppressed by addition of sorbitol to the growth medium (supplementary material Fig. S1A). Cells formed clumps that sedimented rapidly (supplementary material Fig. S1B). The appearance of the triple mutant was strikingly similar to that of the *cla4Δ chs3Δ* cells (Fig. 1G), despite the presence of chitin at bud necks and cell walls, as shown by Calcofluor White staining (Fig. 1G). In fact, the chitin content of this strain was 3.5 times that of wild type (Table 1). The large increase in chitin could be ascribed to the hyperpolarized growth of the mutant (Schmidt et al., 2008). This type of growth also results in weaker cell walls (Schmidt

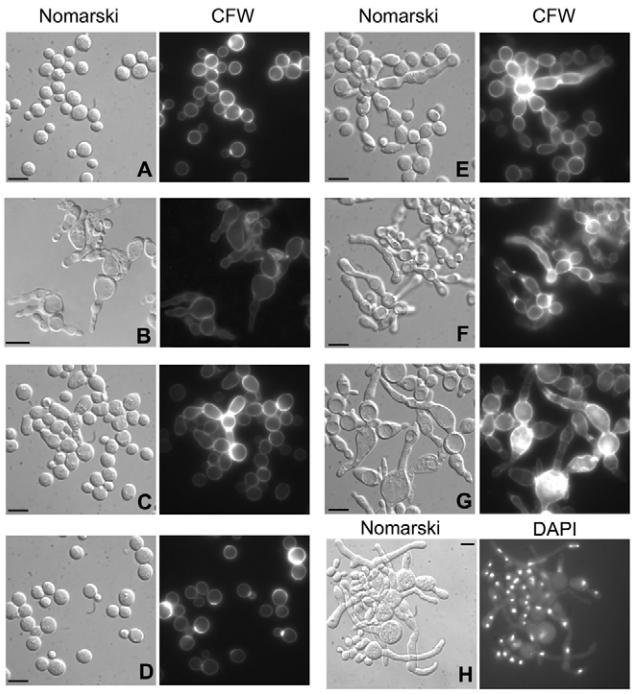

Fig. 1. Double or triple mutants of *CLA4* and the *CRH* genes show aberrant morphology similar to that of *cla4Δ chs3Δ* cells. Morphology of different strains and chitin staining with Calcofluor White (CFW) after growth in YEPD at 25℃. (**A**) Wild type (YPH499); (**B**) *cla4Δ chs3Δ* (YMS90); (**C**) *cla4Δ* (YMS134); (**D**) *crh1Δ crh2Δ* (NBT014); (**E**) *cla4Δ crh1Δ* (NBT011); (**F**) *cla4Δ crh2Δ* (NBT015); and (**G**) *cla4Δ crh1Δ crh2Δ* (NBT017). The exposure for the Calcofluor frames of C and E–G was shorter than for the other frames to compensate for a high fluorescence that would have masked the distribution of chitin. In **H**, staining of nuclei with DAPI is shown. Scale bars: 10 μm.

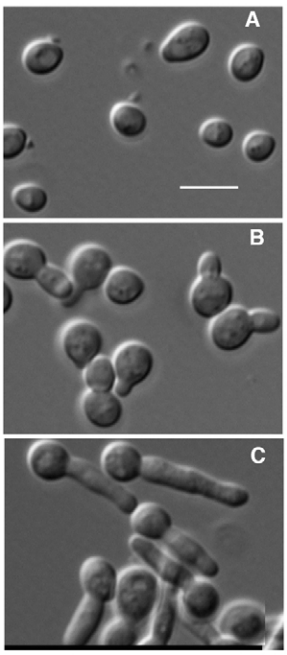

Fig. 2. Round cells of NBT017 (*cla4Δ crh1Δ crh2Δ*) give rise to elongated cells. Round cells were isolated after growth in YEPD-supplemented minimal medium (see Materials and Methods) and transferred to hydroxyurea-containing YEPD. Pictures were taken at 0 time (**A**), after 3.5 hours (**B**) and after 5.5 hours (**C**). Scale bar: 10 μm.

et al., 2008), which explains the extensive lysis we observed at 37℃ and the protection by sorbitol mentioned above.

Despite the morphological abnormalities of the triple mutant, some roundish, apparently normal cells were observed (Fig. 1G). The number of these cells increased when the strain was grown in minimal medium. To investigate the possible presence of two different populations, round cells, unbudded or with very tiny buds, were isolated by sucrose gradient centrifugation (Fig. 2A; see Materials and Methods) and incubated with YEPD in the presence of hydroxyurea, to limit growth to one generation. These cells gave rise to mostly elongated daughter cells (Fig. 2B,C). In an experiment in which the round cells were incubated at different temperatures, 60% of 218 cells yielded elongated buds at 25℃ and 75% of 223 cells at 30℃. These results show that the round cells and elongated cells belong to the same population.

Single deletions of *CRH1* or *CRH2* in a *cla4Δ* background (Fig. 1E,F) led to cells with an intermediate morphology between

Table 1. Chitin content of different strains

Strain	Chitin (% of cell dry weight)
YPH 499 (wild type)	0.83
YMS 134 (*cla4Δ*)	1.3
NBT017 (*cla4Δ crh1Δ crh2Δ*)	2.95

that of *cla4Δ*, which does show some elongated buds (Fig. 1C) and that of the triple mutant (Fig. 1G). Crh2 function seemed to be more important than that of Crh1, because *cla4Δ crh2Δ* cells were considerably more abnormal than *cla4Δ crh1Δ* cells (Fig. 1E,F). This result might be related to the preferential localization of Crh2 at the mother–bud neck (Rodríguez-Peña et al., 2000). The morphological defects of the triple mutant were completely corrected by transformation with a plasmid carrying *CLA4* and partially by plasmids with *CRH1* or *CRH2* (Fig. 3), giving rise to morphologies similar to those of the corresponding double mutants (compare Fig. 3 with Fig. 1). By contrast, plasmids harboring mutations of *CRH1* or *CRH2* in the putative glutamyl proton donor (Carbohydrate-Active Enzyme Database, www.cazy.org) did not complement the defect (Fig. 3), showing that the transglycosylase activity of the Crh proteins is required for correct morphogenesis.

Despite the anomalous morphology, nuclear division seemed to take place efficiently in the triple mutant, as judged from 4′,6-diamidino-2-phenylindole (DAPI) staining (Fig. 1H). The same cannot be said for septin organization and localization. Septin defects have been noted in *cla4* mutants (Cvrcková et al., 1995; Schmidt et al., 2003; supplementary material Fig. S2), but those in the triple mutant *cla4Δ crh1Δ crh2Δ* are more extensive (supplementary material Fig. S2), especially with reference to localization, because septins are often observed in places distant from the mother–bud neck. Because of the importance of septins for cell division (Longtine and Bi, 2003), it was not surprising that often septa were abnormal, as visualized by electron microscopy (Fig. 4I). As in the case of septin mutants (Slater et al., 1985; Roh et al., 2002b), we found instances in which septa were being formed adjacent to a lateral cell wall (Fig. 4J). Wide

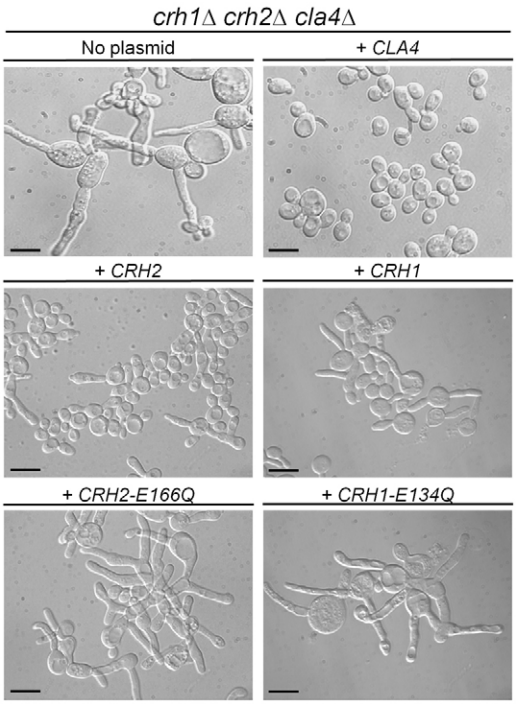

Fig. 3. Mutants in the putative active site of Crh1p and Crh2p do not complement the defects of the *cla4Δ crh1Δ crh2Δ* strain. Complementation of NBT017 (*cla4Δ crh1Δ crh2Δ*) defects with different plasmids. *CLA4* stands for pMS17 (pRS200-*CLA4*), *CRH2* for pNBc13 (YEp352-*CRH2-HA*), *CRH1* for pNBc15 (YEp352-*CRH1-HA*), *CRH2-E166Q* for pNB23 (pNBc13-$CRH2^{E166Q}$) and *CRH1-E134Q* for pNB48 (pNBc15-$CRH1^{E134Q}$). Note in the two bottom frames that a plasmid with a Crh protein mutated in the presumed active site does not complement the defect of the triple mutant. Scale bars: 10 μm.

necks could be observed in the *cla4Δ chs3Δ* and the *cla4Δ crh1Δ crh2Δ* mutants (Fig. 4F,H).

Some of the strains described above contain mutations either in the *CRH* genes or in the *CHS3* gene, but none of them has mutations in both. For a more complete genetic analysis, the phenotype of a *crh1Δ crh2Δ chs3Δ* strain was compared to those of wild type and of *crh1Δ crh2Δ* and *chs3Δ* mutants. Two properties were examined: one, the morphology of the cells and clumped growth as consequence of the *chs3* mutation (Shaw et al., 1991); the other, the sensitivity of the different strains to Congo Red (Rodríguez-Peña et al., 2000). Only the two strains containing the *CHS3* deletion gave rise to clumps during growth (supplementary material Fig. S2). Wild type was sensitive and a *crh1Δ crh2Δ* strain hypersensitive to Congo Red, as previously observed (Rodríguez-Peña et al., 2000). The *chs3Δ* mutant was resistant to Congo Red, as expected and the triple mutant showed the same behavior (supplementary material Fig. S3). Thus, in both cases the introduction of mutations in the *CRH* genes did not affect the phenotype of a *chs3Δ* strain. This was expected, because the chitin made through Chs3 is the substrate for the Crh proteins, therefore in the absence of that chitin the presence or absence of Crh1 and/or Crh2 does not make a difference.

We were also able to obtain a quadruple mutant, *cla4Δ crh1Δ crh2Δ chs3Δ*. Again, the phenotype of this strain is the same as that of *cla4Δ crh1Δ crh2Δ* or *cla4Δ chs3Δ* (supplementary material Fig. S3), comfirming that Crh1 and Crh2 are not operative in the absence of Chs3.

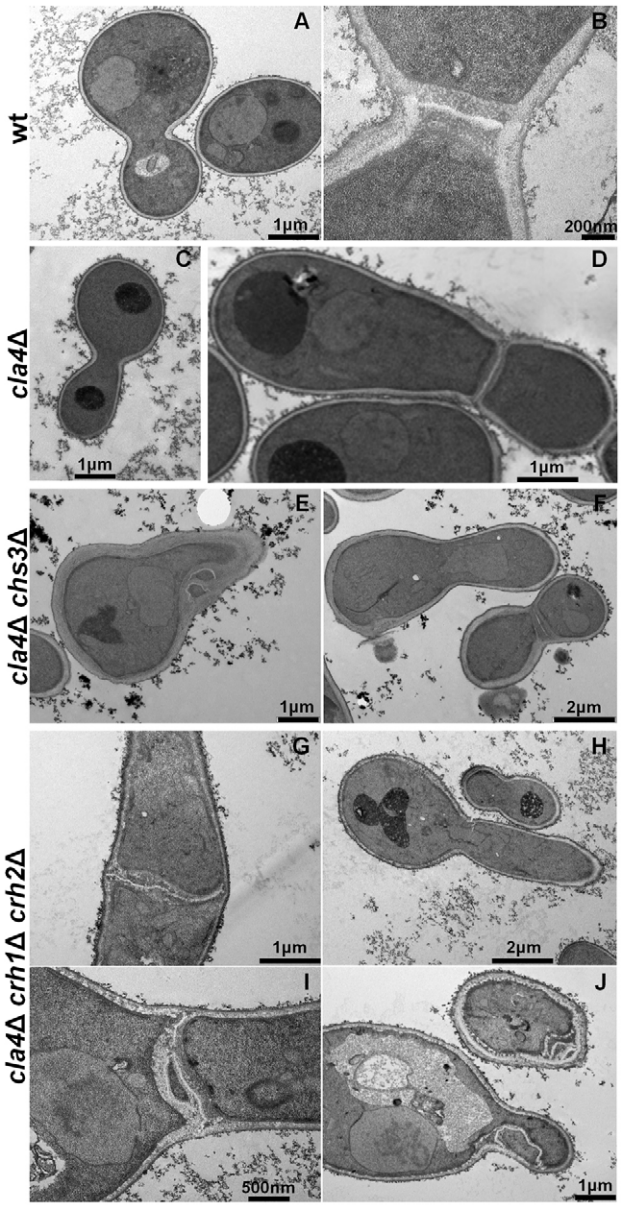

Fig. 4. Electron microscopy of cells of different strains. (**A**) Emerging bud and (**B**) complete septum of the wild-type strain YPH499. (**C**) Emerging bud and (**D**) complete septum of the *cla4Δ* strain YMS134. For examples of abnormal septa in a *cla4Δ* strain (see Schmidt et al., 2003). (**E**) Ectopic septum and (**F**) emerging bud and normal septum in the *cla4Δ chs3Δ* strain YMS90. Ectopic septa were rare in this strain. (**G**) Normal-like but wide septum, (**H**) emerging bud, (**I**) abnormal septum and (**J**) ectopic septa of the *cla4Δ crh1Δ crh2Δ* strain NBT017. Note that emerging buds are wider in YMS90 and NBT017 than in YPH499 or YMS134.

Neck widening is independent from hyperpolarized growth and occurs during bud development

The first effect observed in *cla4Δ* cells incubated with nikkomycin Z was widening of the mother cell-bud neck (Schmidt et al., 2003). Events at the neck give rise to further

abnormal morphology, because washing out Nikkomycin Z at the small bud size did not prevent bud elongation (Schmidt et al., 2003). The bud elongation observed in the triple mutant probably results from the neck widening, which leads to disorganization of the septin ring and subsequent activation of the morphogenesis checkpoint (Lew, 2003). Here, the protein kinase Swe1 becomes activated and inactivates, by phosphorylation, the master kinase of the cell cycle, Cdc28, leading to arrest of the cycle and persistent apical growth. To suppress that growth, we deleted *SWE1* in the triple mutant. As expected, the length of the buds decreased dramatically (Fig. 5), although not completely; however, the necks remained wide (see also below). This result clearly shows that neck widening is independent from polarized growth.

Because of the effect of sorbitol on the growth of the triple mutant (supplementary material Fig. S1), it was of interest to determine its influence upon the morphology of the different strains. The appearance of wild-type (YPH499) and *cla4Δ* (YMS134) cells was not perturbed by growth in the presence of 0.8 M sorbitol, except for some general shrinkage. However, with the *cla4Δ crh1Δ crh2Δ* mutant (NBT017), a decrease in elongated buds and a concomitant increase in roundish cells was detected (Fig. 5). A similar effect could be observed in the *cla4Δ chs3Δ* mutant (YMS90) (Fig. 5). In the *cla4Δ crh1Δ crh2Δ swe1Δ* strain (ECY108), which already had shorter buds, little change was seen in the presence of sorbitol (Fig. 5).

Because of the crucial importance of events at the neck, we measured neck diameter in the different strains (see Materials and Methods for the procedure). There was a small increase in diameter going from wild type to a *cla4Δ* mutant (Fig. 6), which was however significant, according to a *t*-test, with a $P<0.001$. The necks of the triple mutant were much wider, in fact even wider than those of the *cla4Δ chs3Δ* strain (Fig. 6), whereas those of the triple mutant deleted for *SWE1* showed only a small decrease relative to NBT017. Interestingly, although growth in the presence of sorbitol led in general to a small decrease in neck width, the ratio between the values of the different mutants and those of *cla4Δ* remained about the same, indicating that the effect of sorbitol is not specific (Fig. 6).

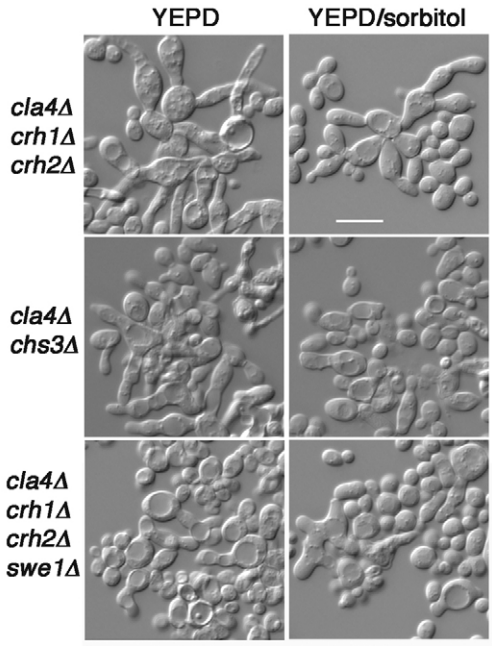

Fig. 5. *SWE1* deletion and addition of sorbitol to the medium reduce bud elongation. *cla4Δ crh1Δ crh2Δ* is NBT017, *cla4Δ chs3Δ* is YMS90 and *cla4Δ crh1Δ crh2Δ swe1Δ* is ECY108. Cells were grown at 25℃ in YEPD or YEPD containing 0.8 M sorbitol. Scale bar: 10 μm.

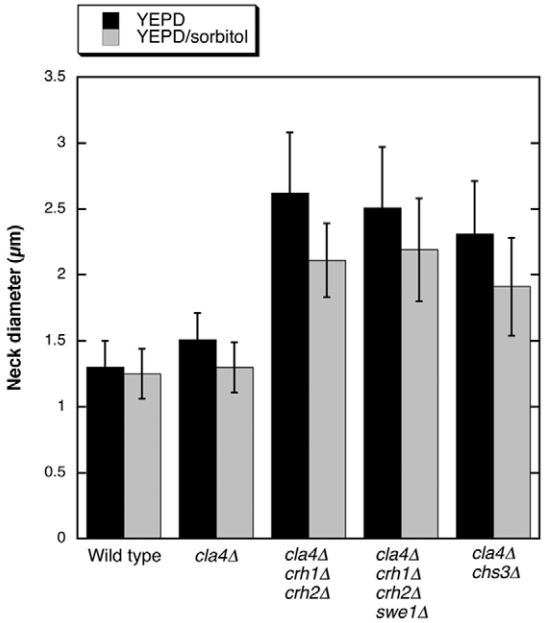

Fig. 6. Mother–bud neck diameters are enlarged in cells with defects in both septins and chitin–glucan crosslinks. The strains used were YPH499 (wild type), YMS134 (*cla4Δ*), NBT017 (*cla4Δ crh1Δ crh2Δ*), ECY108 (*cla4Δ crh1Δ crh2Δ swe1Δ*) and YMS90 (*cla4Δ chs3Δ*). The number of cells measured, from left to right in the figure, was 120, 108, 166, 134, 133, 95, 103, 105, 185 and 135. Standard deviation is shown. For technical details of the measurements, see Materials and Methods. Cells were grown in YEPD at 25℃, with the addition of 0.8 M sorbitol where indicated.

A question that came up is whether the neck in the triple mutant is already wide at the beginning of bud emergence or if it widens during bud development. To answer this question, we used the experiment of Fig. 2. Neck diameters were measured at the small bud stage (Fig. 2B) and again after the buds had completely developed (Fig. 2C). At the first stage, the value was 2.2±0.23 μm (s.d.; 102 cells), at the final stage 2.68±0.35 μm (110 cells). The difference was highly significant with $P<<0.001$ by the *t*-test. Given that one can measure neck diameters only when a bud, albeit small, is fully formed, the measured difference is a minimal value.

In summary, lack of linkages between the chitin ring and β(1-3)glucan at the mother–bud neck lead to neck growth and consequent widening.

Inhibition of the Crh proteins in a *cla4Δ* mutant causes morphological aberrations

In the experiments described above, linkage of chitin to β(1-3)glucan was abolished by deletion of *CHS3* or of *CRH1* and *CRH2*, whereas in earlier work it was done by inhibition of Chs3 with Nikkomycin Z (Schmidt et al., 2003). It is not known how the deletants suppress lethality in the presence of the *cla4*

mutation, and in that sense an inhibition experiment would be more straightforward. Thus, it seemed desirable to test the effect of a Crh inhibitor on a *cla4Δ* strain. We previously found that chitin oligosaccharides inhibited the transfer of chitin to sulforhodamine-labeled β(1-3)-linked glucose oligosaccharides, presumably by interfering with Crh1 and Crh2 activity (Cabib et al., 2008). Indeed, when a *cla4Δ* strain was grown in the presence of triacetylchitotriose, pronounced morphological aberrations were observed. However, staining with Calcofluor White showed loss of fluorescence at the neck, suggesting that it was the loss of chitin, rather than of its linkage to β(1-3)glucan, that caused those changes. Further work with the use of different mutants indicated that the chitin loss was associated with the presence or Crh1 in the cell. We used therefore a *cla4Δ crh1Δ* strain, which does not appear to lose chitin, as detected with Calcofluor White, in the presence of moderate concentrations of triacetylchitotriose (Fig. 7A–D). When chitin was labeled *in vivo* with ^{14}C-glucosamine (Cabib and Durán, 2005) and the distribution of chitin was determined, a pronounced decrease in the percentage of chitin bound to β(1-3)glucan was detected in the presence or triacetylchitotriose (supplementary material Table S2). Upon incubation of single cells of this strain with the oligosaccharide, pronounced neck widening and bud elongation were observed (Fig. 7E,F; Table 2). The differences in neck diameter, although significant, are reduced by the fact that the strain is already abnormal because of the *crh1* mutation. These results show that inhibition of the Crh proteins has similar effects to the deletion of the corresponding genes.

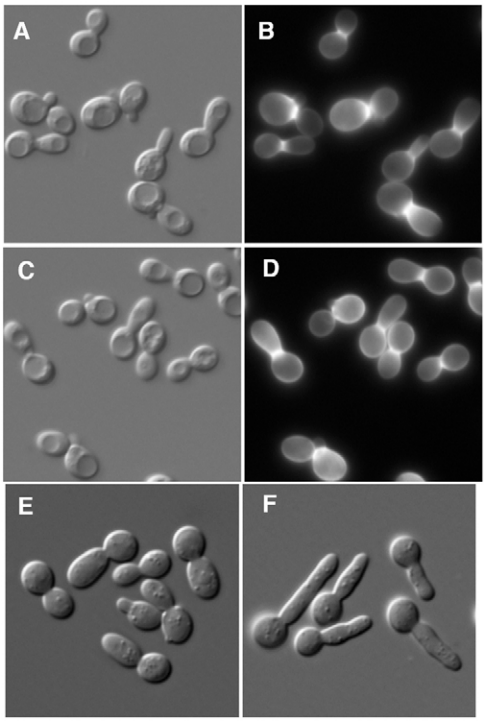

Fig. 7. Incubation of NBT012 (*cla4Δ crh1Δ*) with triacetylchitotriose leads to bud elongation. All pictures were taken with Nomarski optics, except for (B) and (D), which show chitin fluorescence after staining with Calcofluor White. Unbudded cells were isolated by sucrose gradient centrifugation and incubated at 25°C in YEPD supplemented with 0.1 M hydroxyurea for 3 hours (**A–D**) or, in a separate experiment, for 4 hours (**E,F**). In A, B and E, there were no additions; in C, D and F, 2 mM chitooligosaccharide was added. As shown in B and D, the addition of oligosaccharide had little effect on chitin staining, but caused bud elongation (E,F). Addition of 4 mM oligosaccharide caused a similar effect, but with greater elongation (supplementary material Table S2).

Discussion

Our results clearly show that loss of Crh1 and Crh2 function in a strain partially defective in septin ring organization leads to dramatic morphological changes (Fig. 1). Deletion of *CRH1* and *CRH2* in a wild-type background has no effect in the morphology (Fig. 1D) and that of *CLA4* causes only minor defects (Fig. 1C). The morphological aberrations observed in deletion mutants of the Crh proteins are caused by a loss of the chitin transferase activity in those strains, because mutations in the putative active site lead to the same outcome (Fig. 3). The aspect of the *crh1Δ crh2Δ cla4Δ* cells is practically undistinguishable from that of a *chs3Δ cla4Δ* mutant, which completely lacks the chitin ring (Fig. 1B,G). Given that the triple mutant has plenty of chitin (Table 1) and also shows chitin rings (Fig. 1G), it must be concluded that the mere presence of a chitin ring is not sufficient for the control of morphogenesis. It is necessary that the chitin be attached to glucan, in agreement with our hypothesis. The primary defect in the triple mutant is clearly located at the mother–bud neck. Not only is the neck of the mutant much wider than that of wild type, but it also remains so when the bud elongation is largely eliminated by deletion of *SWE1* (Figs 5, 6). In addition to deletion of *SWE1*, another factor that partially limits bud elongation is the presence of an osmotic protector, sorbitol, in the growth medium (Fig. 5). The mechanism of this effect is not clear. However, it is important to note that necks remain wide in the presence of sorbitol (Fig. 6). This supports our interpretation that crosslinks interfere with growth, and is evidence against one suggesting that they mechanically increase the rigidity of the cell wall. If this were the case, sorbitol should prevent neck widening by neutralizing the turgor pressure of the cell.

The neck diameter in the *crh1Δ crh2Δ cla4Δ* and in the *chs3Δ cla4Δ* strains, although large, is smaller than those observed in a *cla4Δ* mutant after exposure to Nikkomycin Z (Schmidt et al., 2003) and so is the increase in diameter during budding. In our view, the explanation for this difference is that in the Nikkomycin experiment there was no time for the emergence of mechanisms to circumvent the damage, and in fact the cells died (Schmidt et al., 2003). However, the mutants survived by the activation of some suppressor, and they presumably could do so by limiting growth at the neck to some degree. This remedy was only partial and had stochastic effects, as shown by the variability in morphology during growth of the mutants, and in particular by

Table 2. Effect of triacetylchitotriose on bud length and neck diameter of NBT012 (*cla4Δ crh1Δ*)

	Ratio length:width[a,b]	Neck diameter (μm)[b]
Control	1.67±0.79 (113 cells)	1.75±0.19 (116 cells)
2 mM oligosaccharide	2.25±1.07 (117 cells)	1.94±0.18 (103 cells)
4 mM oligosaccharide	2.6±1.3 (124 cells)	2.05±0.19 (99 cells)

Results are ±s.d.

[a]The ratio is a measure of bud elongation. The length was measured from the neck to the bud tip.

[b]All differences between control and incubation with oligosaccharide are significant at $P<0.001$ by Student's *t*-test.

the presence of round cells, which however, gave rise to elongated cells after budding (Fig. 5). We could avoid the genetic or regulatory changes by growing the cells in the presence of a chitin oligosaccharide, which inhibits Crh1 and Crh2 and caused neck widening and bud elongation (Fig. 7). In this case, we had to use a *cla4Δ crh1Δ* strain, because of the finding that triacetylchitotriose elicits a strong chitinase activity in Crh1. This effect also occurs, albeit at a reduced level, with Crh2 (M. Mazán, N.B., Z. Zemková, J.A. and V. Farkas, unpublished data); therefore, it was necessary to use the oligosaccharide at a moderate concentration, with less dramatic effects than those shown by Nikkomycin Z.

From our earlier results (Schmidt et al., 2003) and those of the present study, it is clear that the chitin ring and the septin ring have a redundant function in the control of neck growth. The two rings apparently work independently and certainly by different mechanisms. The need to maintain the integrity of the neck region, where cytokinesis takes place, for cell survival, might have led to development of two parallel systems for its control, during evolution. Although the two rings seem to act independently, there is a relationship between them: Chs3, the synthase responsible for the formation of the chitin ring, is linked to the septin ring through Chs4, Bni4 and Glc7 (De Marini et al., 1997; Kozubowski et al., 2003). Thus, formation of the septin ring ensures correct localization of the chitin ring. This function appears to be maintained in *cla4Δ* mutants, which have normal chitin rings.

With our previous results and those presented here, all three predictions of our hypothesis for growth control at the mother–bud neck have been verified: (1) the chitin ring at the neck is specifically attached to β(1-3)glucan (Cabib and Durán, 2005); (2) the β(1-3)glucan linked to chitin has the properties expected from a component of a quiescent cell wall (Cabib et al., 2012); (3) from the findings of the present study it can be concluded with certainty that the linkage of chitin to β(1-3)glucan and not just the presence of the chitin ring, is necessary for the control of growth at the mother–daughter neck. This conclusion would be valid even if the chitin ring had some mechanical stabilizing function at the neck. Thus, here is a straightforward case in which a chemical linkage between two molecules has an essential role in the control of morphogenesis. It would not be surprising if nature had used this device in other systems.

Materials and Methods

Strains and growth

The strains used are listed in supplementary material Table S3. Cells were grown in YEPD (2% glucose, 2% peptone, 1% yeast extract) or in minimal medium (2% glucose, 0.7% yeast nitrogen base) plus requirements, unless indicated otherwise. Synthetic complete (SC) medium (Rose et al., 1990) was used in some cases.

Strain construction

To obtain the single *crh2Δ* and double *cla4Δ crh2Δ* mutants, the *CRH2* ORF was deleted in YPH499 or YMS134 as previously described (Cabib et al., 2012). To obtain the single *crh1Δ* and double *cla4Δ crh1Δ* mutants, the *CRH1* ORF was replaced in YPH499, YMS134 or YMS306 strains as already outlined (Cabib et al., 2012). Finally, to generate the triple mutant *cla4Δ crh1Δ crh2Δ*, the *CRH2* ORF was replaced in the double mutant *cla4Δ crh1Δ*, by using the His3MX6 module (Rodríguez-Peña et al., 2000). Correct replacement was verified using the same primers described previously.

To obtain the *chs3Δ*, *chs3Δ crh1Δ crh2Δ* and *crh1Δ crh2Δ cla4Δ chs3Δ* mutants in the YPH499 background, the *CHS3* ORF was replaced in wt, *crh1Δ crh2Δ* and *crh1Δ crh2Δ cla4Δ* strains, respectively, by the geneticin resistant marker (KanMX4 module), using the Short Flanking Homology PCR technique (Rodríguez Peña et al., 1998) and the following primers: 5′-GGTCCTGTTTAGACTATCCGCAGGAAAGAAATTAGAATGCGTACGCTGCAGGTCGAC-3′ and 5′-CATACTGTCTATGCAACGAAGGAGTCACTTTCCTCCTTCCGATCGATGAATTCGAGCTCG-3′. Correct ORF replacement was verified by PCR using the following primer pairs: 5′-GCACGTACTACGTAGCCAC-3′ and 5′-CCGTGCGGCCATCAAAATG-3′; 5′-ACGGCCACATCAAATACCC-3′ and 5′-GCCCAGATGCGAAGTTAAG-3′.

The *swe1::KanMX4* deletion cassette was amplified by PCR as previously described (Schmidt et al., 2003) and the PCR product was used to transform NBT017, yielding strain ECY108. Strain YMS90, containing both a *CHS3* and a *CLA4* deletion, was obtained by transforming strain ECY46-4-1B (*chs3::LEU2*; Crotti et al., 2001) with a *cla4::URA3* deletion cassette described by Schmidt and colleagues (Schmidt et al. 2003).

Plasmid construction

Plasmids pNB23 and pNB48 were obtained from plasmids pNBc13 and pNBc15, respectively (Cabib et al., 2007). Both plasmids were used as templates for PCR site-directed mutagenesis using the QuikChange Site-Directed Mutagenesis Kit (Agilent Technologies, Stratagene, La Jolla, CA). To obtain plasmid pNB23, primers 5′-CCGGCGCAGGTGATCAACTTGATTACGAATTCG-3′ and 5′-CGAATTCGTAATCAAGTTGATCACCTGCGCCGG-3′ were used to bring about the change E166Q in the *CRH2* gene. To construct plasmid pNB48, primers 5′-GTGAGATTTGGATCAAATTGATATTGAATGGG-3′ and 5′-CCCATTCAATATCAATTTGATCCAAATCTCAC-3′ were used to effect the change E134Q in the *CRH1* gene. Primer design and PCR conditions were as suggested by the manufacturer. The presence of the designed mutation as well as the absence of additional mutations within the construction were verified by DNA sequencing. Plasmids used in this study are listed in supplemental material Table S4.

Phenotypic analysis

Yeast cells were grown overnight in YEPD liquid media at 24℃ to mid-log phase. The cultures were then diluted in YEPD to 3×10^6 cells ml^{-1} and incubated at 24℃ for an additional 3 hours. Five µl ($\sim15\times10^3$ cells) plus five 1:5 serial dilutions were spotted on YEPD solid media and YEPD plus 0.5 M Sorbitol. Growth was monitored on the plates after 2 days at 24℃ and 37℃, respectively.

Microscopic analysis

Yeast cells were grown at 24℃ overnight in YEPD, SC-URA (for cells bearing the plasmids pLA10, pNBc13, pNB23, pNBc15 or pNB48) or SC-LEU (for cells bearing the pMS17 plasmid). The cultures were then diluted in YEPD to 3×10^6 cells ml^{-1} and maintained at 24℃ or 37℃ for an additional 3 hours. After this time, cells were collected, washed with PBS and analyzed before and after staining with Calcofluor White, DAPI or propidium iodide.

For Calcofluor White staining, cells were stained with Fluorescent brightener 28 (Sigma-Aldrich, St Louis, MO, final concentration: 50 mg ml^{-1} in PBS) for 10 minutes. Cells were washed twice with PBS and suspended in a suitable volume of the same buffer. For staining of nuclear DNA, cells were fixed in 70% ethanol for 15 minutes, then washed twice with PBS and incubated with DAPI (final concentration 50 ng ml^{-1}) for 5 minutes. Cells were then washed twice with PBS and resuspended in this buffer. For analysis of cell lysis, yeasts were stained with propidium iodide (0.05 mg ml^{-1}), as previously described (de la Fuente et al., 1992).

Nonstained cells were visualized by DIC. Stained yeast cells were analyzed by fluorescence microscopy and DIC, using a Nikon TE2000 fluorescence inverted microscope equipped with CCD (Melville, NY). Digital images were acquired with an Orca C4742-95-12ER camera (Hamamatsu Photonics, Japan) and processed with the Imaging Aquacosmos or Hamamatsu HCImage Imaging systems software. Alternatively, images were obtained with a Zeiss Axioskop microscope equipped with a Retiga Exi camera and analyzed with iVision software.

For *CDC10-GFP* localization, yeast cells transformed with pLA10 (Cid et al., 1998), were grown as explained above, washed twice with PBS and visualized with the same fluorescence microscope.

Genetic analysis

YMS90 was mated to YPH499-B followed by sporulation and tetrad dissection as described (Rose et al., 1990). For spore germination, plates were incubated at 25℃. Segregants were identified by nutritional requirements and morphology.

Electron microscopy

For transmission electron microscopy, samples were prepared as previously described (Gómez-Esquer et al., 2004). Basically, cells were grown overnight at 28℃ to mid-log phase in YEPD liquid media. The culture was diluted to 3×10^6 cells ml^{-1} in YEPD and incubated at 28℃ for an additional few hours until a concentration of 2×10^7 cells ml^{-1} was reached. Then, 1 ml of cell suspension was collected and washed with PBS. Cells were fixed with 500 µl of fixative solution (0.05 M sodium cacodylate buffer, pH 7.2, 2% paraformaldehyde, 1.5% glutaraldehyde) at 4℃ overnight. The cells were subsequently washed three times with PBS, treated with 1% (w/v) potassium permanganate for 90 minutes at room temperature and washed again with PBS three times. After washing, cells were dehydrated through an ethanol gradient (30, 40, 50, 60, 70, 80, 90 and 100%, v/v; 10 minutes each) and embedded in Epon 812 resin (Electron Microscopy Sciences) following the manufacturer's

instructions. The solid product was cut into ultrathin slices, stained with uranyl acetate and examined with a Zeiss EM902 electron microscope.

Single-cell experiments

For the budding of round cells of strain NBT017 (*crh1Δ crh2Δ cla4Δ*, Fig. 2) the strain was grown at 25℃ in minimal medium to which 10% YEPD was added. Under these conditions, the occurrence of single round cells was relatively high and the doubling time was 3–3.5 hours, whereas in minimal medium growth was extremely slow and in YEPD few round cells were found. Single round cells were isolated by centrifugation in sucrose gradients as previously reported (Drgonová et al., 1999) and incubated at 25℃ or 30℃ in YEPD, in the presence of 100 mM hydroxyurea. At different times, digital images of cells were acquired and measurements were made.

In the experiments in which triacetylchitotriose was used to inhibit the Crh proteins (Fig. 7), NBT012 (*cla4Δ crh1Δ*) was grown in YEPD at 25° and unbudded cells were isolated as outlined above. The unbudded cells were incubated at 25℃ in YEPD containing different concentrations of the chitooligosaccharide and 100 mM hydroxyurea, and the morphology was observed and recorded at different times.

Chitin distribution in the absence and presence of triacetylchitotriose

Chitin was labeled *in vivo* with ^{14}C-glucosamine as described (Cabib and Durán, 2005) either with no addition (control) or in the presence of 2 mM triacetylchitotriose. Cell walls were prepared, treated with alkali and solubilized by carboxymethylation before or after treatment with β(1-6)glucanase, as previously outlined (Cabib and Durán, 2005). Chitin distribution was determined with the curdlan method (Cabib, 2009).

Measurement of mother–bud neck diameter

The measurement of neck diameter was done on phase contrast photographs obtained with a Zeiss Axioskop microscope and a Retiga Exi digital camera, with the measuring tool of the iVision software. Because of the aberrant morphology of some of the strains used, it was often difficult to identify newly emerged buds. To facilitate their recognition, we stained the cells with fluorescein-conjugated Concanavalin A (Tkacz et al., 1971) and let them grow for about one generation. The new buds are easily distinguished from older cells, because their surface is not fluorescent.

Congo Red sensitivity

To determine the sensitivity of the different strains to Congo Red, cells were grown overnight in YEPD media at 30℃ to mid-log phase. Cultures were diluted in YEPD to 3×10^6 cells ml^{-1} and 5 µl plus five 1:5 serial dilutions were spotted on YEPD solid media and YEPD plus Congo Red. Growth was monitored on the plates after 2 days at 30℃.

Chitin determination

Chitin was measured in cell walls as previously described (Cabib and Sburlati, 1988). Results were referred to cell dry weight.

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References

Cabib, E. (2009). Two novel techniques for determination of polysaccharide cross-links show that Crh1p and Crh2p attach chitin to both β(1-6)- and β(1-3)glucan in the *Saccharomyces cerevisiae* cell wall. *Eukaryot. Cell* **8**, 1626-1636.

Cabib, E. and Bowers, B. (1975). Timing and function of chitin synthesis in yeast. *J. Bacteriol.* **124**, 1586-1593.

Cabib, E. and Durán, A. (2005). Synthase III-dependent chitin is bound to different acceptors depending on location on the cell wall of budding yeast. *J. Biol. Chem.* **280**, 9170-9179.

Cabib, E. and Sburlati, A. (1988). Enzymatic determination of chitin. *Methods Enzymol.* **161**, 457-459.

Cabib, E., Bowers, B., Sburlati, A. and Silverman, S. J. (1988). Fungal cell wall synthesis: the construction of a biological structure. *Microbiol. Sci.* **5**, 370-375.

Cabib, E., Roh, D.-H., Schmidt, M., Crotti, L. B. and Varma, A. (2001). The yeast cell wall and septum as paradigms of cell growth and morphogenesis. *J. Biol. Chem.* **276**, 19679-19682.

Cabib, E., Blanco, N., Grau, C., Rodríguez-Peña, J. M. and Arroyo, J. (2007). Crh1p and Crh2p are required for the cross-linking of chitin to β(1-6)glucan in the *Saccharomyces cerevisiae* cell wall. *Mol. Microbiol.* **63**, 921-935.

Cabib, E., Farkas, V., Kosík, O., Blanco, N., Arroyo, J. and McPhie, P. (2008). Assembly of the yeast cell wall. Crh1p and Crh2p act as transglycosylases in vivo and in vitro. *J. Biol. Chem.* **283**, 29859-29872.

Cabib, E., Blanco, N. and Arroyo, J. (2012). Presence of a large β(1-3)glucan linked to chitin at the Saccharomyces cerevisiae mother-bud neck suggests involvement in localized growth control. *Eukaryotic Cell* **11**, 388-400.

Caudron, F. and Barral, Y. (2009). Septins and the lateral compartmentalization of eukaryotic membranes. *Dev. Cell* **16**, 493-506.

Cid, V. J., Adamíková, L., Cenamor, R., Molina, M., Sánchez, M. and Nombela, C. (1998). Cell integrity and morphogenesis in a budding yeast septin mutant. *Microbiology* **144**, 3463-3474.

Crotti, L. B., Drgon, T. and Cabib, E. (2001). Yeast cell permeabilization by osmotic shock allows determination of enzymatic activities *in situ. Anal. Biochem.* **292**, 8-16.

Cvrcková, F., De Virgilio, C., Manser, E., Pringle, J. R. and Nasmyth, K. (1995). Ste20-like protein kinases are required for normal localization of cell growth and for cytokinesis in budding yeast. *Genes Dev.* **9**, 1817-1830.

de la Fuente, J. M., Alvarez, A., Nombela, C. and Sánchez, M. (1992). Flow cytometric analysis of *Saccharomyces cerevisiae* autolytic mutants and protoplasts. *Yeast* **8**, 39-45.

DeMarini, D. J., Adams, A. E. M., Fares, H., De Virgilio, C., Valle, G., Chuang, J. S. and Pringle, J. R. (1997). A septin-based hierarchy of proteins required for localized deposition of chitin in the *Saccharomyces cerevisiae* cell wall. *J. Cell Biol.* **139**, 75-93.

Drgonová, J., Drgon, T., Roh, D.-H. and Cabib, E. (1999). The GTP-binding protein Rho1p is required for cell cycle progression and polarization of the yeast cell. *J. Cell Biol.* **146**, 373-387.

Gómez-Esquer, F., Rodríguez-Peña, J. M., Díaz, G., Rodríguez, E., Briza, P., Nombela, C. and Arroyo, J. (2004). *CRR1*, a gene encoding a putative transglycosidase, is required for proper spore wall assembly in *Saccharomyces cerevisiae*. *Microbiology* **150**, 3269-3280.

Hayashibe, M. and Katohda, S. (1973). Initiation of budding and chitin-ring. *J. Gen. Appl. Microbiol.* **101**, 295-301.

Kollár, R., Reinhold, B. B., Petráková, E., Yeh, H. J. C., Ashwell, G., Drgonová, J., Kapteyn, J. C., Klis, F. M. and Cabib, E. (1997). Architecture of the yeast cell wall. β(1—>6)-glucan interconnects mannoprotein, β(1—>3)-glucan, and chitin. *J. Biol. Chem.* **272**, 17762-17775.

Kozubowski, L., Panek, H., Rosenthal, A., Bloecher, A., DeMarini, D. J. and Tatchell, K. (2003). A Bni4-Glc7 phosphatase complex that recruits chitin synthase to the site of bud emergence. *Mol. Biol. Cell* **14**, 26-39.

Lew, D. J. (2003). The morphogenesis checkpoint: how yeast cells watch their figures. *Curr. Opin. Cell Biol.* **15**, 648-653.

Lippincott, J. and Li, R. (1998). Sequential assembly of myosin II, an IQGAP-like protein, and filamentous actin to a ring structure involved in budding yeast cytokinesis. *J. Cell Biol.* **140**, 355-366.

Longtine, M. S. and Bi, E. (2003). Regulation of septin organization and function in yeast. *Trends Cell Biol.* **13**, 403-409.

Orlando, K., Sun, X., Zhang, J., Lu, T., Yokomizo, L., Wang, P. and Guo, W. (2011). Exo-endocytic trafficking and the septin-based diffusion barrier are required for the maintenance of Cdc42p polarization during budding yeast asymmetric growth. *Mol. Biol. Cell* **22**, 624-633.

Rodriguez-Peña, J. M., Cid, V. J., Sánchez, M., Molina, M., Arroyo, J. and Nombela, C. (1998). The deletion of six ORFs of unknown function from *Saccharomyces cerevisiae* chromosome VII reveals two essential genes: *YGR195w* and *YGR198w. Yeast* **14**, 853-860.

Rodríguez-Peña, J. M., Cid, V. J., Arroyo, J. and Nombela, C. (2000). A novel family of cell wall-related proteins regulated differently during the yeast life cycle. *Mol. Cell. Biol.* **20**, 3245-3255.

Roh, D.-H., Bowers, B., Riezman, H. and Cabib, E. (2002a). Rho1p mutations specific for regulation of β(1—>3)glucan synthesis and the order of assembly of the yeast cell wall. *Mol. Microbiol.* **44**, 1167-1183.

Roh, D.-H., Bowers, B., Schmidt, M. and Cabib, E. (2002b). The septation apparatus, an autonomous system in budding yeast. *Mol. Biol. Cell* **13**, 2747-2759.

Rose, M. D., Winston, F. and Hieter, P. (1990). Methods in yeast genetics. *A Laboratory Course Manual*. Cold Spring Harbor, NY: Cold Spring Harbor Press.

Schmidt, M., Bowers, B., Varma, A., Roh, D.-H. and Cabib, E. (2002). In budding yeast, contraction of the actomyosin ring and formation of the primary septum at cytokinesis depend on each other. *J. Cell Sci.* **115**, 293-302.

Schmidt, M., Varma, A., Drgon, T., Bowers, B. and Cabib, E. (2003). Septins, under Cla4p regulation, and the chitin ring are required for neck integrity in budding yeast. *Mol. Biol. Cell* **14**, 2128-2141.

Schmidt, M., Drgon, T., Bowers, B. and Cabib, E. (2008). Hyperpolarized growth of *Saccharomyces cerevisiae cak1*^{P212S} and *cla4* mutants weakens cell walls and renders cells dependent on chitin synthase 3. *FEMS Yeast Res.* **8**, 362-373.

Shaw, J. A., Mol, P. C., Bowers, B., Silverman, S. J., Valdivieso, M. H., Durán, A. and Cabib, E. (1991). The function of chitin synthases 2 and 3 in the Saccharomyces cerevisiae cell cycle. *J. Cell Biol.* **114**, 111-123.

Sikorski, R. S. and Hieter, P. (1989). A system of shuttle vectors and yeast host strains designed for efficient manipulation of DNA in Saccharomyces cerevisiae. *Genetics* **122**, 19-27.

Slater, M. L., Bowers, B. and Cabib, E. (1985). Formation of septum-like structures at locations remote from the budding sites in cytokinesis-defective mutants of Saccharomyces cerevisiae. *J. Bacteriol.* **162**, 763-767.

Tkacz, J. S., Cybulska, E. B. and Lampen, J. O. (1971). Specific staining of wall mannan in yeast cells with fluorescein-conjugated concanavalin A. *J. Bacteriol.* **105**, 1-5.

Versele, M. and Thorner, J. (2004). Septin collar formation in budding yeast requires GTP binding and direct phosphorylation by the PAK, Cla4. *J. Cell Biol.* **164**, 701-715.