UNIVERSITÄT OSNABRÜCK

Crosstalk of signaling pathways for cell wall integrity, cytokinesis and glucose metabolism in the milk yeast *Kluyveromyces lactis*

Dissertation

Zur Erlangung des akademischen Grades

Doctor rerum naturalium

(Dr. rer. nat.)

Fachbereich Biologie/Chemie der Universität Osnabrück

Vorgelegt von

Dorthe Rippert

Osnabrück, Mai 2016

Erstgutachter: Prof. Dr. Jürgen J. Heinisch

Abteilung Genetik, Universität Osnabrück

Zweitgutachter: Prof. Dr. Hans Merzendorfer

Abteilung Molekulare Physiologie, Universität Siegen

Table of contents

1. Summary

This thesis was dedicated to the investigation of the interplay between signaling pathways governing cell wall biosynthesis, central carbohydrate metabolism and cytokinesis in two different yeast species. The first part of the thesis addressed cell wall biosynthesis, which forms an essential structure and determines both shape and integrity of fungal cells and hyphae. It also serves as a first barrier against changing and adverse environmental conditions. Cell wall synthesis and remodeling is primarily mediated by the cell wall integrity pathway in both the budding yeast *Saccharomyces cerevisiae* and the milk yeast *Kluyveromyces lactis*. The latter is a Crabtree-negative yeast with a similar life cycle to that of *S. cerevisiae*. Yet, *K. lactis* did not undergo a whole genome duplication, presenting a lower degree of genetic redundancy, which often avoids the need for multiple gene deletions. In this work the SNF1 kinase complex was identified as playing a role in cell wall synthesis. It belongs to the family of AMP-activated protein kinases (AMPKs), whose primary function is thought to be the regulation of energy balance in different organisms. Mutants with defects in this complex have a thinner cell wall than wild type and are hypersensitive to cell wall stress agents such as Caspofungin, Calcofluor white and Congo red. Epistasis analyses with mutants affecting cell wall integrity signaling suggested a parallel action of CWI- and SNF1-signaling in the two yeast species. Further genetic analyses indicated a known downstream effector of the SNF1 kinase complex, the transcriptional repressor Mig1, to mediate the signaling function in cell wall synthesis, too. Further epistasis analyses indicated that the hypersensitivity of the SNF1 complex mutants to the stress agents can be suppressed by an additional defect in the upper part of glycolysis. This has been attributed to the accumulation of the glycolytic intermediates glucose-6-phosphate and fructose-6-phosphate, which serve as precursors of cell wall polysaccharides. A function of the SNF1 complex in yeast cell wall synthesis has not been described, until now.

In order to study the relation to cytokinesis, i.e. the last step of cell division, first some tools to follow this process in *K. lactis* had to be established in the second part of the thesis. Cytokinesis is also an essential feature of life since it ensures cell proliferation. In yeast and mammalian cells the concluding abscission of the plasma membrane is initiated by the construction of an actomyosin ring (AMR), accompanied by the formation of a primary septum, and followed by the deposition of secondary septa from both mother and daughter cells. Cytokinesis is a highly coordinated process and regulation is not yet fully explained. Proteins important for cytokinesis in *S. cerevisiae* were identified in *K. lactis* by *in silico* analyses and then genetically investigated. In contrast to *S. cerevisiae*, analysis of deletion mutants showed that deletion of the gene encoding *K. lactis* myosin II (*KlMYO1*) as a key

component of the AMR is viable but temperature-sensitives and therefore dispensable for cytokinesis in *K. lactis* under normal growth conditions. Also different from its *S. cerevisiae* ortholog, a *Klcyk3* deletion is lethal, while *inn1* deletions are not viable in either yeast species. In contrast to the other genes studied, expression of *INN1* does not cross-complement between the two yeast species, which could be narrowed down to a species specificity of the C2 domain in the Inn1 protein, which activates the chitin synthase II in *S. cerevisiae*. Deletions lacking the *K. lactis* chitin synthase II gene (*KlCHS2*) are also not viable. Fluorescently tagged versions of all proteins show a similar spatiotemporal localization at the bud neck as their counterparts in *S. cerevisiae*. The second part of this work thus provides insights into the regulation of cytokinesis in *K. lactis* and indicates that AMR constriction and its regulation could be more important in *K. lactis* than in *S. cerevisiae,* while the overall sequence of morphological events in cytokinesis is quite similar.

2. Introduction

2.1 *Kluyveromyces lactis* as a model yeast

Within the last two decades, *K. lactis* has been established as a model yeast besides the well-known budding yeast *S. cerevisiae*. *K. lactis* was named in honor of the microbiologist Albert Jan Kluyver (1888-1995) and was first isolated from milk. Accordingly, it is able to utilize lactose as a sole carbon source, which makes *K. lactis* an important microorganism in dairy industries. Due to the presence of a lactose permease and its cytoplasmic ß-galactosidase activity, it is frequently employed to remove the disaccharide, e.g. for the sake of lactose-intolerant patients.

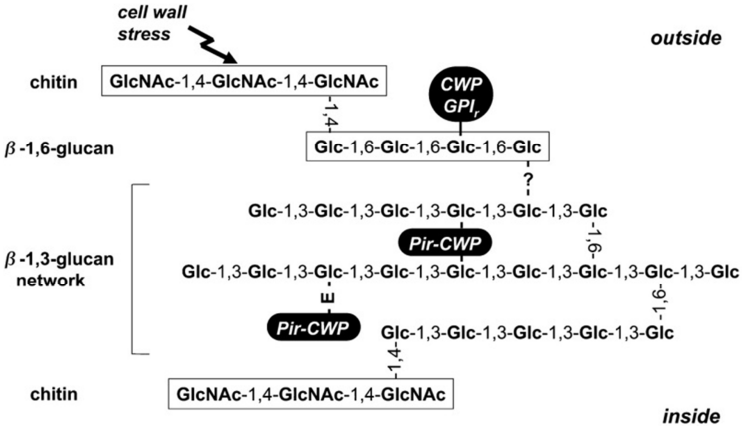
Like *S. cerevisiae*, *K. lactis* belongs to the group of endoascomycetous yeasts. The fact that it disposes of two mating types and that it proliferates by budding both in the stable haploid and the semistable diploid state makes *K. lactis* accessible to classical genetic techniques by crossing, sporulation and tetrad analysis (Schaffrath and Breunig, 2000; Rodicio and Heinisch, 2013). That is why *K. lactis*, along with the fission yeast *Schizosaccharomyces pombe*, is one of the best-studied so-called “nonconventional yeasts”. For the manipulation of genes in a relatively constant genetic background, a congenic strain series was obtained, which is based on the strain CBS2359 whose entire genome has been sequenced (Heinisch *et al.,* 2010; http://genome.jgi.doe.gov/Klula1/Klula1.home.html). A set of cloning vectors with different selectable markers (Chen, 1996) and plasmids carrying markers for PCR-based gene deletions are available (Heinisch *et al.*, 2010). However, the efficiency of homologous recombination is much lower in *K. lactis* than in *S. cerevisiae*, which is mostly due to non-homologous recombination (Kooistra *et al.*, 2004). To improve the frequency of homologous recombination, a deletion of the *KlKU80* gene, whose product is involved in non-homologous end-joining, has been obtained and introduced into the congenic strain background (Kooistra *et al.*, 2004; Heinisch *et al.*, 2010).

K. lactis clearly differs from *S. cerevisiae* in its central carbohydrate metabolism: While *S. cerevisiae* is Crabtree-positive and widely known for its fermentative capacity (González-Siso *et al.*, 2000), *K. lactis* belongs to the Crabtree-negative yeasts and displays the Pasteur effect, i.e. fermentation is repressed under aerobic growth conditions and respiration is the primary route of energy production (Breunig *et al.*, 2000). Previous results have shown, that *K. lactis* has not undergone a whole genome duplication (WGD) as *S. cerevisiae* (ÓhÉigeartaigh *et al.*, 2011). *K. lactis* has only six chromosomes (designated A-F) with an estimated genome size of 12 Mb (Schaffrath and Breunig, 2000). This results in a lower redundancy of genes in *K. lactis* as opposed to *S. cerevisiae* and facilitates genetic and

physiological analyses, since phenotypes are more readily observed upon single gene deletions, where two or more paralogs would have to be deleted in *S. cerevisiae*.

2.2 Similarities between *K. lactis* and *S. cerevisiae* in cell wall composition and signaling

Despite the fact that the milk yeast *K. lactis* has a thinner cell wall than the budding yeast *S. cerevisiae* (Backhaus *et al.*, 2010), its composition is quite similar. In general, the yeast cell wall contributes 10-30% to the cellular dry weight and contains approximately 5% proteins and 95% polysaccharides (Klis *et al.*, 2002). The polysaccharides are composed of 40% mannoproteins, which are covalently bound to cell wall proteins, 60% of long 1,3-ß-glucan chains connected to shorter 1,6-ß-glucan chains, and 1-2% chitin, which is mainly found at the bud neck and in the bud scars, but some is also found in the lateral walls of the mother cell (Fig. 1; Klis *et al.*, 2002). Images from transmission electron microscopy have shown that the cell wall displays a two-layered structure in both *S. cerevisiae* and *K. lactis* (Backhaus *et al.*, 2010). The inner and less electron-dense layer surrounds the plasma membrane and probably contains most of the glucan network, whereas the outer and more electron-dense layer contains more mannoproteins (Klis *et al.*, 2002; Backhaus *et al.*, 2010).



Macromolecule	wall dry wt. (%)
Mannoproteins	35-40
ß-1,6-glucan	5-10
ß-1,3-glucan	50-55
Chitin	1-2

Figure 1: Molecular organization of the yeast cell wall (Levin, 2011) and cell wall macromolecules in *S. cerevisiae* (Klis *et al.*, 2002).

The glucan content of the *K. lactis* cell wall do not differ between cells grown on glucose or ethanol, whereas the mannan content decreases in ethanol grown cells and in the stationary growth phase on glucose (Backhaus *et al.*, 2010). *Vice versa*, the mannan content of the *S. cerevisiae* cell wall increases under stress conditions and glucan shows a tendency to decrease during growth on ethanol and in stationary phase (Backhaus *et al.*, 2010). Both yeast species show a similar amount of chitin under all tested conditions, which contributes little more than 1% to the total cell wall polysaccharides.

In *S. cerevisiae* cell wall synthesis is governed by the cell wall integrity (CWI) signaling pathway (Levin, 2005; Jendretzki *et al.*, 2011). Five plasma membrane spanning sensors (Wsc1, Wsc2, Wsc3, Mid1 and Mtl1) detect the cell surface stress and activate the small GTPase Rho1 by stimulating its GDP to GTP nucleotide exchange via the guanine nucleotide exchange factors (GEFs) Rom1 and Rom2. Rho1 then physically interacts with the protein kinase C (Pkc1) to trigger activation of a conserved mitogen activated protein kinase (MAPK) cascade, which is comprised of the MAPKKK Bck1, the two redundant MAPKKs Mkk1 and Mkk2 and the MAPK Slt2 (synonymous to Mpk1; Levin, 2005). Activated Slt2 is shuttled into the nucleus and triggers the expression of genes involved in cell wall synthesis by activation of the MADS box transcription factor Rlm1 (Jung *et al.*, 2002). Slt2 also phosphorylates the SBF transcription factor, which is a dimer of Swi4 and Swi6 and regulates cell cycle progression (Madden *et al.*, 1997; Baetz *et al.*, 2001). The CWI signaling pathway is conserved in *K. lactis*. Homologues for most of the CWI pathway components have been identified, but there are several differences regarding the impact of specific pathway components as judged from the phenotype of the respective deletion mutants (Backhaus *et al.*, 2011).

The less redundant genome of the milk yeast encodes only three sensor proteins (KlWsc1, KlWsc2/3 and KlMid2). KlWsc1 is a homologue of ScWsc1 with an overall amino acid identity of 39%. KlWsc2/3 is similar to both ScWsc2 and ScWsc3 with an overall identity of approximately 34% to each. KlMid2 is the ortholog of ScMid2 with 41% amino acid identity (Rodicio *et al.*, 2008b). In contrast to *S. cerevisiae*, single deletions of the sensor encoding genes in *K. lactis* do not display any obvious phenotype, whereas the double and triple deletions are severely impaired for growth on rich medium (Rodicio *et al.*, 2008b). Only one functional GEF, KlRom2, which regulates the CWI pathway, was found in *K. lactis* with an overall amino acid identity of 57% to ScRom2 (Lorberg *et al.*, 2003). The gene encoding the *K. lactis* homologue *KlRHO1* has been cloned by heterologous hybridization with a *ScRHO1* probe (Rodicio *et al.*, 2006). In yeast two-hybrid experiments KlRho1 interacts with its downstream target KlPck1 (Rodicio *et al.*, 2006). KlRho1 and KlPck1 are both essential as are their counterparts in *S. cerevisiae* (Rodicio *et al.*, 2006). *K. lactis* homologues of the MAPK module components have also been found and display a high degree of similarity in their protein sequence, too. Among them, the MAPKKK KlBck1 seems to play a less important role in *K. lactis* than in *S. cerevisiae*, since deletion or overexpression of *KlBCK1* does not show any drastic effect (Jacoby *et al.*, 1999). A single gene encoding the MAPKK KlMkk1 has been studied in our laboratory and the deletion strain is sensitive to various cell wall stresses (unpublished results). In contrast to the *Klbck1* deletion, a *Klmpk1* deletion grows poorly at elevated temperatures, as would be expected from a CWI mutant. The

deduced Slt2/Mpk1 sequences of *K. lactis* and *S. cerevisiae* display 85% amino acid identity and all functional domains are highly conserved (Kirchrath *et al.*, 2000). The MADS box transcription factor KlRlm1 is a homolog of the well-described CWI transcriptional activator ScRlm1 from *S. cerevisiae* and was studied in a PhD thesis (Meyer, 2014). The Rlm1 proteins of both yeasts share similar domain structures. Yet, deletion mutants behave differently in their response to cell wall stress agents. Whereas the *Klrlm1* deletion is sensitive to cell wall stressors as expected, the *Scrlm1* deletion is not sensitive but rather hyper-resistant to Congo red and Calcofluor white (Watanabe *et al.*, 1995; López-García *et al.*, 2010). A cross-complementation between the two yeast species was also not found, which indicates that the proteins are indeed functional homologs with regard to CWI signaling, but display a high degree of species specificity (Meyer, 2014).

2.3 Glucose signaling and the SNF1 kinase complex

2.3.1 Physiological role of the SNF1 kinase

One of the most important features of all cells is their ability to adapt to environmental changes, such as the availability of nutrients, the presence of hormones or harmful compounds and changes in temperature or medium pH. With regard to carbon source utilization, glucose repression is found in most microorganisms, including yeasts. Thus, *S. cerevisiae* is known for its capacity to ferment a variety of sugars and to grow on a range of non-fermentable carbon sources, such as glycerol or ethanol. The trimeric Snf1 kinase is a major player in the yeast cell response to glucose availability. Its subunit Snf1 is the founding member of the family of AMP-activated protein kinases (AMPK). The human AMPK serves as a metabolic stress sensor that turns off biosynthetic processes when the cellular ATP concentration drops, which is accompanied by an increase in the concentration of 5'-AMP (Stapleton *et al.*, 1996). It has been suggested that AMPK is a key developmental regulator involved in maintaining energy homeostasis in cells and tissues and may therefore be a good target for treatment of various diseases like Type 2 diabetes, obesity, metabolic syndrome and related heart diseases (Hardie, 2004; Hardie, 2007).

A mutant in the kinase subunit of the SNF1 complex was first found as *cat1* (for catabolite repression) in a screen for mutants which failed to derepress genes required for the utilization of alternative carbon sources (Zimmermann *et al.*, 1977). Later on, a similar screen for cells unable to grow on sucrose (due to the derepression defect) and accordingly renamed as "sucrose non fermenters" (hence *snf1*) yielded new mutant alleles at the same locus (reviewed in Hedbacker and Carlson, 2008). When grown on glucose as a sole carbon

source, *snf1* mutants fail to accumulate glycogen as a storage carbohydrate and compared to wild-type cells has lower levels of glucose-6-phosphate (Huang *et al.*, 1997).
Beside its primary role in response to nutrient stress, SNF1 also responds to several other environmental stresses, such as high external sodium chloride concentrations, heat shock, alkaline pH, and oxidative stress (Hong and Carlson, 2007). In addition, it was also found to be involved in the regulation of sporulation and meiosis, aging, invasive growth in haploid and pseudohyphal growth in diploid cells (Honigberg and Lee, 1998; Ashrafi *et al.*, 2000; Cullen and Sprague, 2000; Hedbacker and Carlson, 2008; Kuchin *et al.*, 2002).

2.3.2 Structure and regulation of the SNF1 kinase complex

The yeast SNF1 is a heterotrimeric complex composed of the catalytic α-subunit Snf1, the regulatory γ-subunit Snf4 and either of three alternative β-subunits Sip1, Sip2 or Gal83, mediating subcellular localization. As expected, the catalytic subunit Snf1 shows the highest degree of cross-species conservation, especially in the N-terminal half of the protein, which contains the kinase domain. This domain harbors a conserved threonine residue at position 210 in its activation loop. Phosphorylation of Thr210 by upstream kinases is required for Snf1 kinase activity. The C-terminal half of the protein comprises a regulatory region, carrying an auto-inhibitory sequence (AIS) and a domain mediating interaction with the ß-subunits (ß-SID, Fig. 2; Amodeo *et al.*, 2010). The γ-subunit Snf4 consists of four consecutive cystathionine-beta-synthase (CBS) motifs (Fig. 2; Amodeo *et al.*, 2010). Tandem pairs of these CBS motifs are called “Bateman domains”, known to bind adenosine derivatives. It has been demonstrated that ADP binding to Snf4 protects the Snf1 subunit from dephosphorylation at Thr210. It has been suggested that ADP is the metabolic signal for activation of the SNF1 kinase in response to glucose starvation (Mayer *et al.*, 2011). This is probably also true for the mammalian homologs, in which the designation as "AMP-activated protein kinase (AMPK)" may thus be misleading. For yeast growing on glucose, the catalytic N-terminal domain of Snf1 is folded back onto the C-terminal autoinhibitory domain. Upon glucose depletion, Snf4 binds to the AIS domain and counteracts this inhibition (Hedbacker and Carlson, 2008).
Each of the ß-subunits may function as the scaffold for heterotrimeric formation. Their C-terminal halves contain a region which interacts with both the α- and the γ-subunits in the complex. At the N-terminus of each ß-subunit a glycogen binding domain (GBD) is located, which has been assigned the function of a carbohydrate binding module (CBM, Fig.2; Amodeo *et al.*, 2010). The N-termini of the three alternative ß-subunits vary between each other, probably to mediate the different subcellular localizations of the different SNF1

complexes. In glucose media all complexes are apparently cytoplasmic. Yet, upon glucose depletion they redistribute depending on the nature of the bound ß-subunit. Snf1 complexes carrying Sip1 localize to the vacuole, whereas complexes with Sip2 remain cytoplasmic, and those with Gal83 translocate to the nucleus (Hedbacker and Carlson, 2008; Vincent *et al.*, 2001). Nevertheless, there appears to be some overlap in function, since the presence of one ß-subunit is required for kinase activity and only the triple deletion (*sip1 sip2 gal83*) displays the *snf1* phenotypes, i.e. it cannot derepress invertase gene expression, grows poorly on alternative carbon sources and fails to phosphorylate the target transcription factors Mig1 and Sip4 (Schmidt and McCartney, 2000).

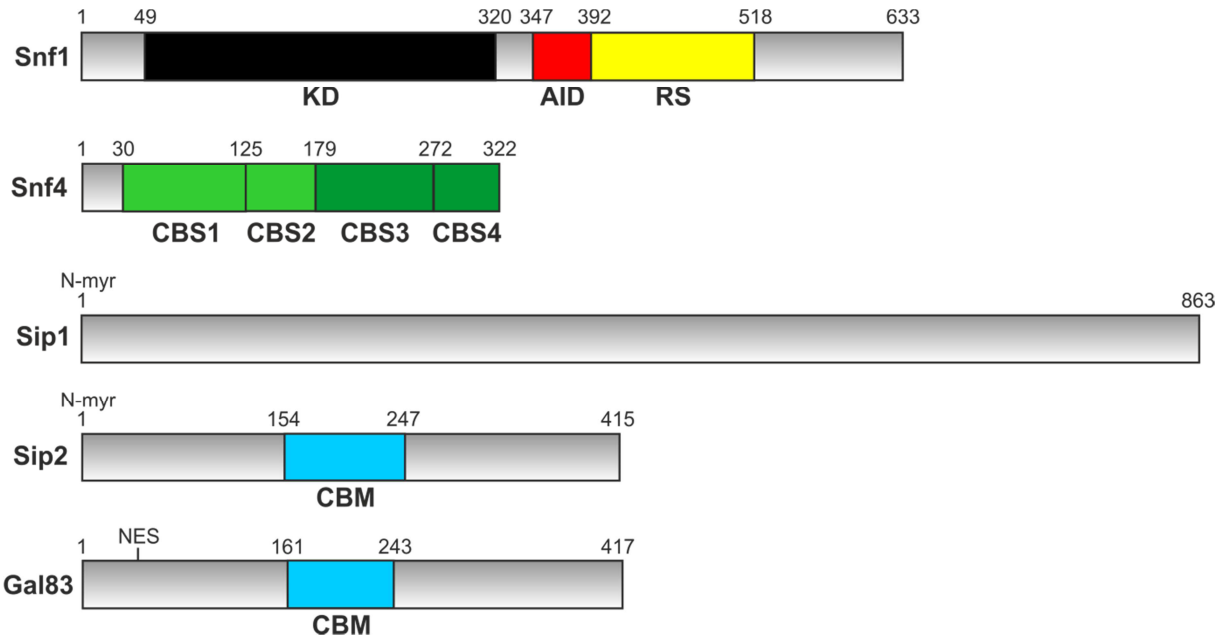


Figure 2: Domain structure of the different subunits of the SNF1 kinase (modified from Amodeo *et al.*, 2010 and Hedbacker and Carlson, 2008). Snf1: protein kinase domain (KD) is shown in black, auto-inhibitory domain (AID) in red and the regulatory sequence (RS) in yellow. Sip2/Gal83: carbohydrate binding module (CBM) is shown in cyan. Snf4: cystathionine-beta-synthase (CBS) domains are shown in green. N-myr = N-myristoylation, NES = nuclear export signal.

As mentioned above, the SNF1 kinase complex is activated upon phosphorylation of Thr210 in the activation loop of the α-subunit. This is mediated by either of three upstream kinases, namely Sak1, Elm1 and Tos3. Each of them may contribute differently to the regulation of the SNF1 kinase (Hong and Carlson, 2007): Sak1 is the major activating kinase and forms a stable complex with SNF1, whereas Elm1 and Tos3 associate more transiently (Hedbacker and Carlson, 2008). The SNF1 kinase complex can be inactivated by dephosphorylation by the dimeric Reg1-Glc7 protein phosphatase (PP1), in which Reg1 serves as a targeting device for the catalytic subunit Glc7. Accordingly, *reg1* deletions lead to a constitutively activated SNF1 kinase complex, and cells lack the ability for glucose repression (Hedbacker and Carlson, 2008). When glycogen synthesis is required, the PP2A-like protein

phosphatase Sit4 was also shown to dephosphorylate Snf1 (Ruiz *et al.,* 2011). It is now generally believed that the activity of SNF1 is primarily determined by the dephosphorylation reaction, meaning that an increased concentration of phosphorylated Snf1-Thr210 basically is caused by a reduced rate of dephosphorylation (Rubenstein *et al.*, 2008; Mayer *et al.*, 2011).

2.3.3 SNF1 target proteins

Given that the SNF1 kinase complex plays an important role in different metabolic processes, it targets a large number of effector proteins. A major target is the transcriptional repressor Mig1, which controls transcription of genes required for the utilization of alternative carbon sources such as sucrose, galactose or mannose (Hedbacker and Carlson, 2008). When glucose is available, Mig1 resides in the nucleus and inhibits the expression of genes for alternative carbon sources, after binding the corepressor complex Ssn6-Tup1. Upon glucose depletion, Mig1 is phosphorylated by the SNF1 complex, which leads to the translocation of Mig1 to the cytoplasm and therefore releases repression of the target genes (DeVit *et al.*, 1997; Smith *et al.*, 1999). In addition to Mig1, several other transcriptional activators like Adr1, Cat8, Sip4 and Rds2 also get phosphorylated by the SNF1 complex (Hedbacker and Carlson, 2008; Soontorngun *et al.*, 2012). In the case of Adr1, this promotes its binding to chromatin in the absence of glucose, activating the expression of genes required for ethanol and glycerol utilization. Cat8, Sip4 and Rds2 are members of the zinc cluster family of transcription factors. Phosphorylated Cat8 and Sip4 bind to carbon source responsive elements (CSRE) found in the promoters of gluconeogenic genes and those encoding glyoxylate cycle enzymes (Schüller, 2003; Rodicio *et al.*, 2008a).

2.3.4 Glucose signaling and its influence on the yeast cell wall

The key function of the SNF1 kinase complex has been assigned to the regulation of glucose signaling in yeast. However, recent investigations also have shown its implication in the regulation of the cell wall synthesis and the CWI pathway.

Induction of *FKS2* gene expression, which encodes a subunit of glucan synthase, in response to glucose depletion is under the control of the SNF1-regulated Mig1 transcriptional repressor (Zhao *et al.*, 1998). Moreover, in transcriptome studies it was shown that the triple deletion *sip1 sip2 gal83* up-regulates a gene cluster, which includes genes for cell wall organization, such as the chitin synthase 3 (encoded by CHS3), the cell wall protein encoding genes *CRH1*, *CWP1* and *YLR194C* (further called *NCW2*, for new cell wall

protein), and also two members of the CWI pathway namely the sensor gene *MID2* and the gene for the MAPK *MPK1/SLT2* (Zhang *et al.*, 2010). In addition, it was presented that the SNF1 complex regulates gene expression for cell wall degrading enzymes (CWDE) in plant pathogenic fungi (Tonukari *et al.*, 2000; Nadal *et al.*, 2010).

2.3.5 SNF1 kinase in the milk yeast *Kluyveromyces lactis*

Glucose repression in *K. lactis* is not as stringent as in *S. cerevisiae*. The accompanying variations in the regulatory networks, which control the utilization of alternative carbon sources, reflect the different physiological properties of *S. cerevisiae* and *K. lactis*.
Nevertheless, glucose repression is also mediated by the SNF1 kinase complex in *K. lactis*, which contains homologs of the subunits described in *S. cerevisiae*. Thus, KlSnf1, first described in mutant studies as Fog2 (for fermentative and oxidative growth), is structurally and functionally equivalent to the catalytic α-subunit Snf1, with an overall amino acid identity of 72% (Goffrini *et al.*, 1996). Only one ß-subunit exists in *K. lactis*, which is most closely related to Gal83 (overall identity 49%) and therefore named KlGal83 (Fog1; Goffrini *et al.*, 1996). A gene for a putative regulatory γ-subunit KlSnf4 (KLLA0F10769; http://genolevures.org/klla.html) can be found in the *K. lactis* genome, but has not been investigated, until now. Except on glucose, *Klsnf1* and *Klgal83* mutants do not grow on various fermentable and non-fermentable carbon sources. However, in contrast to the *snf1* mutant of *S. cerevisiae*, the *Klsnf1* mutant is still able to derepress invertase gene expression and can also grow on sucrose as sole carbon source (Goffrini *et al.*, 1996). It was also shown that the *KlSNF1* gene of *K. lactis* can complement the phenotypes of a *S. cerevisiae snf1* deletion (Goffrini *et al.*, 1996), demonstrating its functional homology.
K. lactis also has several orthologs of target proteins of the SNF1 kinase complex described in *S. cerevisiae*. Although the regulatory cascade thus seems to be evolutionary conserved, the regulatory circuits present some important differences between the two yeast species (Mehlgarten *et al.*, 2015). Heterologous expression studies have shown that the *K. lactis* homologue of *MIG1* complements the corresponding *S. cerevisiae* deletion (Cassart *et al.*, 1995). Four Snf1-dependent phosphorylation sites were identified in *S. cerevisiae* (Ser222, Ser278, Ser311 and Ser381) and two of the key residues (Ser278 and Ser311) are conserved in KlMig1 (Smith *et al.*, 1999). KlMig1 represses structural and regulatory genes within the lactose-galactose regulon in the presence of glucose by regulating *KlGAL1* gene expression (Fig. 3; Dong and Dickson, 1997). As mentioned above expression of the *KlINV1* gene, which encodes an invertase, is not controlled by KlMig1 and glucose repression is maintained even in the absence of KlMig1 (Georis *et al.*, 1999). In *S. cerevisiae*, the *GAL4*

and *CAT8* genes are subject to Mig1-mediated glucose repression, but the promoters of their *K. lactis* orthologs do not present potential binding sites for KlMig1 (Georis *et al.*, 2000), again indicating that their expression is independent of KlMig1. In contrast to the lack of conservation of Mig1-dependent gene expression, the transcriptional activator KlCat8 is still a target of the KlSNF1 complex (Fig. 3).

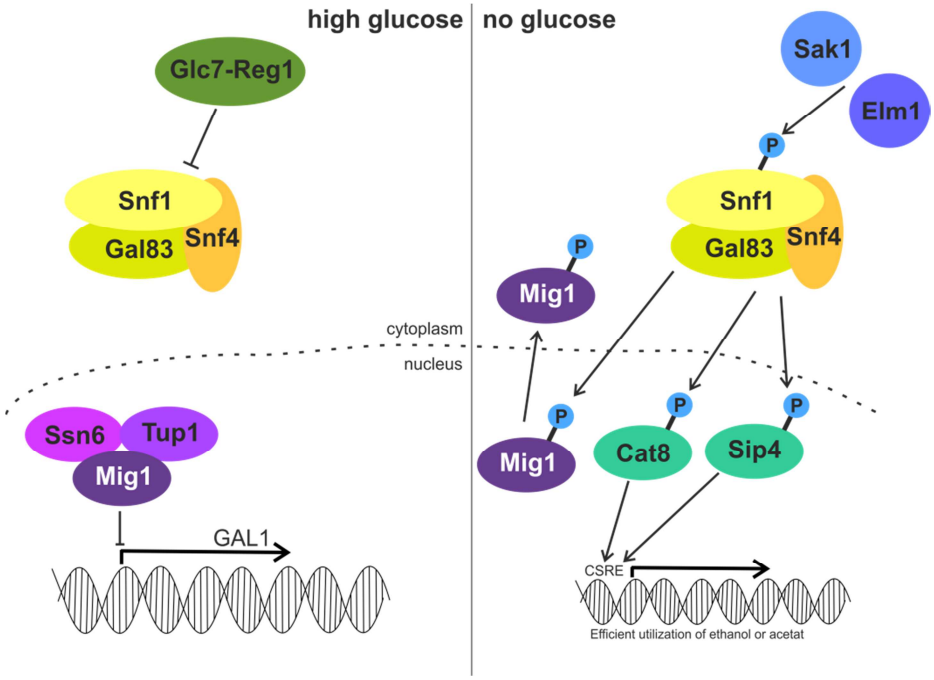


Figure 3: Presumed mode of SNF1 kinase regulation in *K. lactis*.

Although KlCat8 controls the expression of genes encoding enzymes of the glyoxylate cycle, in contrast to its *S. cerevisiae* counterpart, it is not directly involved in the regulation of key genes of gluconeogenesis (Georis *et al.*, 2000; Charbon *et al.*, 2004; Lodi *et al.*, 2001). Recently it was shown, that KlCat8 can bind to carbon source responsive elements (CSREs) in the promoter of the gene encoding the transcriptional activator KlSip4 *in vivo* (Mehlgarten *et al.*, 2015). KlSip4 is also a putative target of the KlSNF1 complex and serves as a key regulator for gluconeogenic gene expression, especially by binding to the CSREs of the *KlICL1* promoter, and for the carnitine shuttle genes (Fig. 3; Rodicio *et al.*, 2008a; Mehlgarten *et al.*, 2015).

2.4 Regulation of cytokinesis

2.4.1 General features of cytokinesis in cells from all biological kingdoms

Cytokinesis is defined as the final process of cell division, which is a fundamental cellular process essential for cell proliferation in all life forms. In this process one cell is separated into two daughter cells through the assembly of new membranes (and in some cases cell wall) after replication and division of their genomic DNA. Except for plant cells, all other life forms (prokaryotes as well as eukaryotes) use a contractile machinery, whose contraction and closure facilitate assembly of new membranes and the completion of cytokinesis (Balasubramanian *et al.*, 2012).

Bacterial cells, which do not have a nucleus, have ancestral homologs of both tubulin (FtsZ) and actin (FtsA; Lutkenhaus *et al.*, 2012). FtsA or ZipA, which contain a transmembrane domain, can tether FtsZ to the membrane and promote the formation of a “Z ring”, which interacts with other conserved proteins in cell division to form a multi-subunit complex called “divisome”. The divisome coordinates FtsZ ring constriction with the assembly of a division septum (Balasubramanian *et al.*, 2012; Lutkenhaus *et al.*, 2012). Plant cell cytokinesis diverges strongly from this mechanism, which has been attributed to the massive cell wall. Instead of a cleavage furrow, which does not exist in plant cells, vesicles from the Golgi are transported along microtubule to mid of the cell during telophase, where they merge to form a cell plate. To this plate vesicles containing cell wall material are continuously attached and mediate its expansion. The orientation and assembly of the cell plate is arranged by the plant-specific cytoskeletal preprophase band (PPB) and a bipolar microtubule array called phragmoplast (McMichael and Bednarek, 2013).

In yeast and mammalian cells the contractile apparatus is composed of an actomyosin ring (AMR). The interaction of actin and myosin produces the contraction of the AMR at the cleavage furrow, which facilitates the assembly of cell membranes and in the case of yeast is accompanied by cell wall synthesis. Details on this process will be discussed in the following chapters.

Since inhibition of cytokinesis causes polyploidy that may render cells tumorigenic in humans, it is important to study the mechanism and regulation of cytokinesis in detail in order to understand the normal cellular function as well as the underlying mechanisms of related human diseases. Since the key components and the general mechanisms of cytokinesis are conserved from yeast to mammalian cells, the genetically tangible budding yeast *S. cerevisiae* is a good model organism to study the coordination and regulation of cytokinesis.

2.4.2 Control of cytokinesis in *S. cerevisiae*

Cytokinesis in the budding yeast *S. cerevisiae* is realized by the coordinated actions of the contractile actomyosin ring (AMR), the targeted membrane deposition and the formation of a primary septum (PS, Fig. 4; Bi and Park, 2012). AMR constriction is closely followed by the centripetal growth of the PS, which after its completion is followed by the synthesis of two secondary septa (SS) at either side of the PS (Fig. 4). Whereas the PS is a chitinous and electron-lucent structure which is only formed during cytokinesis, the SS are more electron-dense and are structurally similar to the lateral cell wall. At the end of cytokinesis cells become separated after degradation of the PS and a portion of the SS by an endochitinase and several glucanases secreted by the daughter cell.

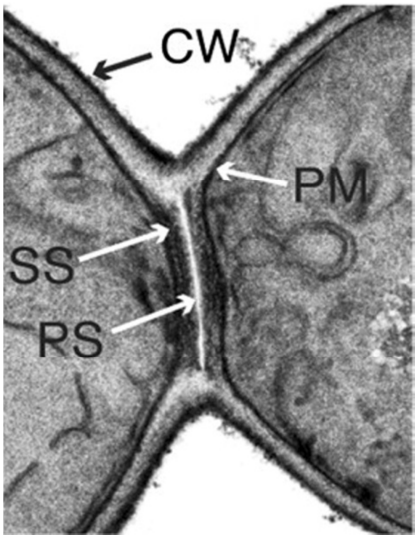


Figure 4: Electron micrograph of the yeast bud neck during cytokinesis (Bi and Park, 2012). CW=cell wall, PM=plasma membrane, PS=primary septum, SS=secondary septa.

2.4.2.1 Septins and their essential role in cytokinesis

Septins belong to a family of GTP-binding and filament-forming proteins that are conserved from yeast to humans, but not in higher plants. They assemble into heterooligomeric complexes as building blocks for higher-order structures such as filaments and rings. Septins were first discovered in 1971 in the budding yeast *S. cerevisiae* during a search for cell cycle mutants defective in cell separation (Hartwell, 1971). *S. cerevisiae* disposes of seven different septins. Two, Spr3 and Spr28, are specific for sporulation, whereas the other five septins (Shs1, Cdc3, Cdc10, Cdc11 and Cdc12) are expressed during vegetative growth (Longtine and Bi, 2003).

These five mitotic septins form distinct rod-shaped, nonpolar octameric complexes with Cdc3, Cdc10 and Cdc12 at their core and Cdc11 replaceable with Shs1 at either end (Fig. 5; Bertin *et al.*, 2008; Bertin *et al.*, 2011). These heterooctamers assemble into parallel filaments by an end-to-end organization of Cdc11, and are paired through the predicted C-terminal coiled-coil domains of Cdc3 and Cdc12 (Fig. 5; Bertin *et al.*, 2008; Booth *et al.*, 2015). Shs1 is non-essential and has been suggested to be an accessory septin, which is most similar to Cdc11 and may replace it in some complexes (Iwase *et al.*, 2007). Shs1-containing complexes assemble into small rings consisting of staggered short filaments. All five septins share a common domain structure, which includes a

phosphoinositide binding motif, a GTP-binding domain, a septin-unique element and a coiled-coiled region at the C-terminal end (with the exception of Cdc10, where the latter is not present; Versele *et al.*, 2004).

Septin filaments are first recruited to the presumptive bud site in late G1 by the activated GTPase Cdc42 and its effectors Gic1 and Gic2 (Gladfelter *et al.*, 2002; Iwase *et al.*, 2005), where they assemble into a cortical ring. After bud emergence the septin ring adapts an hourglass shape spanning the mother-bud neck, which at the onset of cytokinesis splits into two separate rings that sandwich the cytokinesis machinery. These septin rings attract late factors of cytokinesis, such as chitin synthase 2 and secretory vesicles (Gladfelter *et al.*, 2001; Longtine and Bi, 2003). The splitting of the septin ring is controlled by the mitotic exit network (MEN; Cid *et al.*, 2001; Lippincott *et al.*, 2001), which comprises a small GTPase (Tem1), a protein kinase cascade (primarily Cdc15 and Dbf2/Dbf20-Mob1 kinases) and a phosphatase (Cdc14; Balasubramanian *et al.*, 2004; Meitinger *et al.*, 2012).

As expected all five septins are localized at the yeast bud neck during cytokinesis (Versele *et al.*, 2004). This localization of the septins and also their assembly into filaments appear to be essential for cell survival, as demonstrated by mutant analyses. Three of the five septin genes, *CDC3*, *CDC11* and *CDC12,* are essential, whereas *shs1* deletions show only mild morphological defects (Iwase *et al.*, 2007). Although *CDC10* was originally described as an essential gene, *cdc10* deletions show only moderate morphological defects in other laboratory strains of *S. cerevisiae* (Hartwell, 1971; Versele *et al.*, 2004). Yet, double deletions of *shs1* and *cdc10* are not viable. Septins have at least two distinct functions in cytokinesis: Before the onset of cytokinesis, the collar filaments of the hourglass are required for AMR assembly and act as a scaffold to recruit proteins, which are required for the septation process. In addition, the septin double ring functions as a diffusion barrier by preventing diffusion of proteins between mother and daughter cells (Bertin *et al.*, 2008).

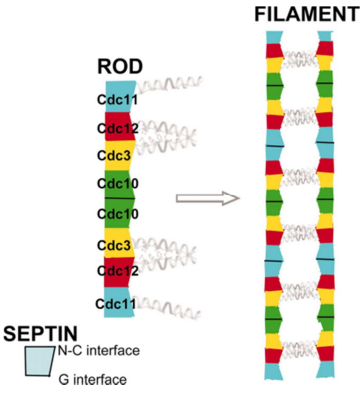


Figure 5: Schematic diagram of the yeast septin rod, filament and paired filament assemblies (Bertin *et al.*, 2008).

2.4.2.2 AMR assembly, constriction and disassembly

One of the first proteins recruited to the septin ring during cytokinesis is the type-II myosin Myo1, which at a later stage associates with actin to form a contractile AMR (Fig. 6; Fang *et al.*, 2010; Wloka and Bi, 2012). Myo1 is recruited to the division site through a biphasic mechanism: From late G1 to the onset of telophase the septin-binding protein Bni5 mediates

the recruitment of Myo1, whereas from the onset of anaphase to the end of cytokinesis this function is carried out by its essential light chain Mlc1 in combination with the IQGAP protein Iqg1 (Fang *et al.*, 2010). At the emerging bud neck Myo1 assembles into a ring-like structure together with F-actin and thus forms the contractile AMR, which is fitted in between the two separated septin rings (Fig. 6). Deletion of *MYO1* is lethal in some genetic backgrounds and causes severe morphological defects in others, such as asymmetric or grossly disoriented primary septa (Fang *et al.*, 2010; Schmidt *et al.*, 2002). This indicates that Myo1 is required for directing AMR assembly and PS formation during cytokinesis (Wloka *et al.*, 2011).

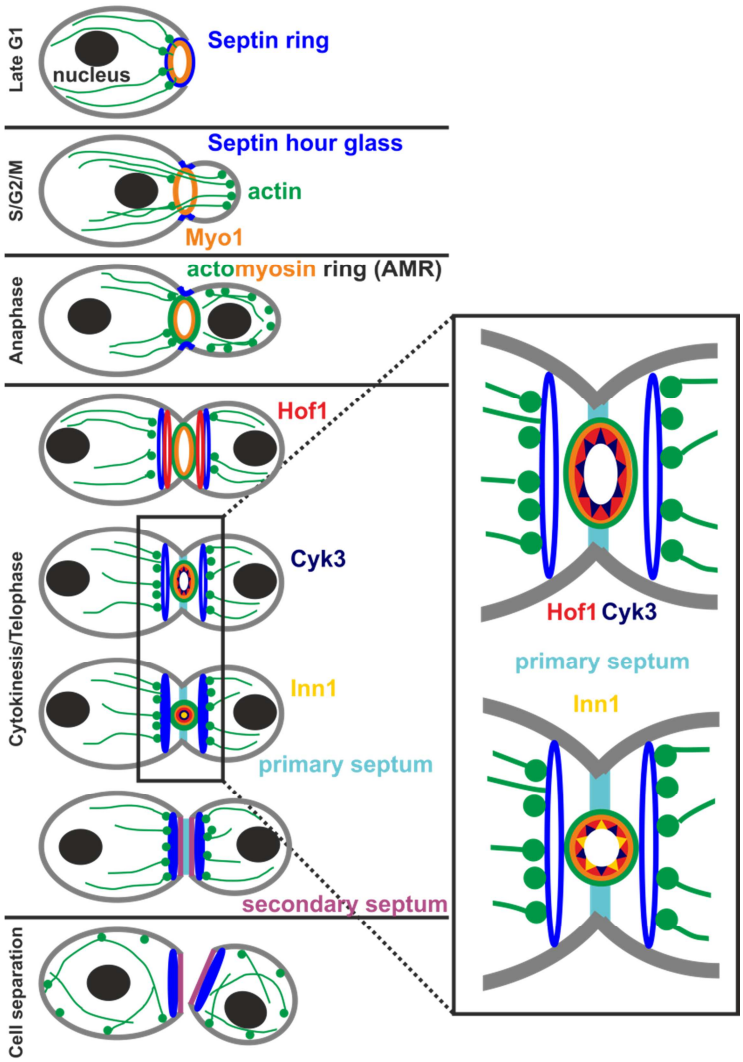


Figure 6: Schematic drawing of cytokinesis in *S. cerevisiae* (modified form Wloka and Bi, 2012).

The constriction of the AMR is controlled by the MEN (Vallen *et al.*, 2000; Lippincott *et al.*, 2001), but it is still unknown how this controlling is mediated. There are three assumptions: i) by controlling septin hourglass splitting, ii) by controlling the PS formation or iii) by directly regulating the AMR components such as Iqg1 (Wloka and Bi, 2012). The question, how the

mechanism of the AMR constriction occurs in cytokinesis, is also not definitely clarified. It was expected that the force for the constriction is generated by a sliding of the myosin motor on actin filaments, but the myosin motor domain is not required for the assembly and constriction of the AMR (Fang *et al.*, 2010; Lord *et al.*, 2005). After completion of the AMR constriction the cytokinetic apparatus is disassembled and the Myo1 signal vanishes from the bud neck. In contrast to AMR constriction, it was reported that the motor domain of Myo1 plays a role in AMR disassembly since Myo1 is defective in disassembly when lacking the entire head domain including the motor domain (Lord *et al.*, 2005; Fang *et al.*, 2010). AMR disassembly is regulated by the anaphase promoting complex (APC), a multi-subunit ubiquitin ligase, which labels specific target proteins for proteosomal degradation (Tully *et al.*, 2009).

2.4.2.3 Septum formation and cell separation

AMR constriction in yeast is accompanied by the formation of a primary septum (Fig. 6), which consists of chitin, a polymer of ß-1,4-linked N-acetylglucosamine. At the bud neck synthesis of this polysaccharide is catalyzed by the chitin synthase isozyme II, which is encoded by the *CHS2* gene. The other two chitin synthase isozymes of *S. cerevisiae* serve different functions (Shaw *et al.*, 1991; Cabib *et al.*, 1993): While Chs1 generally works in cell wall repair including lesions after cell separation (Cabib *et al.*, 1992), Chs3 provides chitin for the bud scar and the minor amounts incorporated into the lateral cell wall (Bulawa, 1993; Cabib and Schmidt, 2003; Lesage and Bussey, 2006). As for the deletions in the genes encoding Cdc10 and Myo1, phenotypes of *chs2* deletions are strain dependent, being lethal in some strains, but viable in others (Cabib and Schmidt, 2003). Viable *chs2* mutants show similar defects in cytokinesis as viable *myo1* deletions, i.e. they lack plasma membrane contraction and do not form a PS. In such strains viability of the *chs2* deletion depends on a functional Chs3, which also localizes to the division site during cytokinesis and can adopt the role of Chs2 by forming a “remedial septum” (Cabib and Schmidt, 2003; Shaw *et al.*, 1991).
Chs2 is synthesized at the rough endoplasmic reticulum (ER) and is held in place as long as it is phosphorylated by Cdk1 (Zhang *et al.*, 2006). In late anaphase Cdk1 becomes inactivated by the MEN signaling cascade and Chs2 gets dephosphorylated by Cdc14, whereby Chs2 enters the secretory pathway and is delivered to the bud neck via the exocytotic machinery (VerPlank and Li, 2005). Phosphorylation of Chs2 by the Dbf2-Mob1 kinase (component of MEN) causes the dissociation from the AMR and thereby its removal from the division site by endocytosis (Oh *et al.*, 2012). After the completion of the PS,

secondary septa (SS) are deposited (Fig. 6), which consists of glucan polysaccharides and several cell wall proteins. Glucans are synthesized by glucan synthases, namely their redundant catalytic subunits Fks1 or Fks2 (Douglas *et al.*, 1994; Inoue *et al.*, 1995; Mazur and Baginsky, 1996). The small GTPase Rho1 serves as a regulatory subunit, and hence has a dual role in CWI signaling and glucan synthesis (Mazur and Baginsky; 1996; Qadota *et al.*, 1996). As stated above, cell separation is mediated by the degradation of the PS and part of the SS by endochitinase and glucanases. Expression of the genes encoding these enzymes is under control of the daughter cell-specific transcription factor Ace2, whose asymmetric localization and activation is governed by the RAM regulatory pathway (regulation of Ace2p activity and cellular morphogenesis; Nelson *et al.*, 2003).

2.4.2.4 Coordination of AMR constriction and PS formation

To ensure an accurate execution of cytokinesis, the spatiotemporal interplay between AMR contraction and the PS formation must be tightly controlled. A plethora of components and regulatory circuits influencing this process have been described in yeast, of which the ones relevant for this thesis have been included in Fig. 6 and will be briefly discussed in the following.

The latest component discovered was Inn1 (required for plasma membrane ingression), which is an essential protein with a N-terminally located C2 ('conserved region 2' of protein kinase C)-like domain and multiple PXXP motifs in its C-terminal region. The latter are mediating the interaction with the SH3 (sarc homology) domains of Hof1 and Cyk3 described below, whereas interaction with Chs2 and the central regulator Iqg1 was found to occur via the C2-like domain of Inn1 (Sanchez-Diaz *et al.*, 2008; Jendretzki *et al.*, 2009; Nishihama *et al.*, 2009). Mutations of two lysines in loop 1 within these domain block cytokinesis underlining its essential role (Devrekanli *et al.*, 2012). *INN1* is an essential gene in most yeast strains (Sanchez-Diaz *et al.*, 2008; Jendretzki *et al.*, 2009) and its lack prevents PS formation in the viable variants (Nishihama *et al.*, 2009). Inn1 activity is modulated in a cell cycle-dependent manner, whereby phosphorylation by Cdc28 (=Cdk1) keeps Inn1 inactive, counteracted by dephosphorylation by Cdc14 (Kuilman *et al.*, 2015; Palani *et al.*, 2012). Consistent with its function as an activator of primary septum formation by Chs2, Inn1 appears at the bud neck for a short time period concomitant with AMR constriction (Jendretzki *et al.*, 2009).

Another protein which appears earlier than Inn1 at the yeast bud neck is Hof1 (Homolog of Cdc fifteen from *S. pombe*). Its localization during cell division is highly dynamic and tightly regulated. It first associates with the septin ring structure prior to the onset of cytokinesis.

Phosphorylation of Hof1 by the MEN kinase complex Dbf2-Mob1 results in the release from the septin ring (Meitinger *et al.*, 2011). Then Hof1 associates and constricts with the AMR before it relocates to the septin collar and separates into two rings (Jendretzki *et al.,* 2009; Meitinger *et al.,* 2012). The Hof1 protein has a F-BAR domain near its N-terminal end and a SH3 domain at the C-terminus, which is required for protein-protein interactions, amongst others with Inn1. A *hof1* deletion is viable, but displays synthetic lethality with either *myo1Δ* or *cyk3Δ* (Vallen *et al.*, 2000), indicating that Hof1 and Cyk3 serve overlapping functions (Korinek *et al.*, 2000). It was recently shown that Rvs167 (the founding member of the BAR domain family) also acts in parallel to Hof1, since the double deletion *hof1 rvs167* is synthetically lethal (Nkosi *et al.*, 2013). Overexpression of Hof1, Cyk3 or Inn1 suppresses the growth defect of the *myo1* deletion, suggesting that they can promote cytokinesis independent of AMR constriction (Ko *et al.*, 2007; Jendretzki *et al.*, 2009).

As mentioned above, Cyk3 (cytokinesis 3) serves overlapping functions with Hof1, and *cyk3* deletions are viable and do not present strong phenotypes. Like Inn1, it resides at the bud neck for a much shorter time period than Hof1. Cyk1 appears at the division site when the AMR starts to constrict and the disappearance of Cyk3 coincides with that of Hof1, which probably marks the time of completion of septum formation (Jendretzki *et al.*, 2009). The protein has a SH3 domain in its N-terminal half and a putative transglutaminase-like domain in its C-terminal half (Nishihama *et al.*, 2009*;* Onishi *et al.*, 2013). Interaction of the PXXP motifs of Inn1 with the SH3 domain of Cyk3 is important for PS formation, since the complex has been proposed to activate chitin synthesis by Chs2 (Nishihama *et al.*, 2009; Meitinger *et al.*, 2010; Meitinger *et al.*, 2011). Overexpression of Cyk3 can rescue the growth defects and failure to form a PS in strains with a viable *inn1* deletion (Nishihama *et al.*, 2009; Oh *et al.*, 2012). Besides its function in promoting PS formation, Cyk3 seems to interact with its transglutaminase-like domain with Rho1-GDP to inhibit its activation at this stage (Onishi *et al.*, 2013). Abnormally activated Rho1 can inhibit PS formation since it promotes SS formation through the MAPK pathway, Fks2 activation and potentially other effectors (Onishi *et al.*, 2013).

2.5 Aims of the thesis

Although the data presented in the introduction clearly show that central carbohydrate metabolism, cell wall synthesis and cytokinesis are interdependent processes in yeast, very few investigations have specifically addressed these relationships. Moreover, the genetic redundancy caused by the whole genome duplication in *S. cerevisiae* frequently leads to the

production of multiple proteins with overlapping functions, thus hindering genetic analyses. Therefore, the following questions were addressed in this thesis:

1. Does the SNF1 complex as a key regulator of central carbohydrate metabolism also regulate cell wall synthesis? - For this purpose, mutants in the genes encoding the complex subunits were constructed both in the model yeast *S. cerevisiae* and in the non-conventional milk yeast *K. lactis*. These mutants were analyzed by transmission electron microscopy for their effect on cell wall structure and employed in epistasis analyses in combination with mutants in the CWI signaling pathway.

2. Are the structural and regulatory components of the cytokinetic machinery conserved between *S. cerevisiae* and *K. lactis* and can the lower degree of genetic redundancy in the latter be exploited to study specific protein functions? - These investigations were based on *in silico* identification of *K. lactis* homologs for the respective *S. cerevisiae* genes, construction of deletion mutants and fluorescently tagged proteins for phenotypic analyses and *in vivo* localization studies, as well as heterologous complementation analyses.

3. Results

3.1 Mutations in SNF1 complex genes affect yeast cell wall strength

K. Backhaus, D. Rippert, C. J. Heilmann, A. G. Sorgo, C. G. de Koster, F. M. Klis, R. Rodicio & J. J. Heinisch,

(2013) *European Journal of Cell Biology* **92**: 383-395

3.2 Mig1 mediates regulation of cell wall synthesis by the SNF1 kinase complex in *Kluyveromyces lactis*

D. Rippert, K. Backhaus, S. Walter, R. Rodicio & J. J. Heinisch,

(2016) *manuscript in preparation*

3.3 Regulation of cytokinesis in the milk yeast *Kluyveromyces lactis*

D. Rippert, N. Heppeler, S. Albermann, H.-P. Schmitz & J. J. Heinisch,

(2014) *Biochimica et Biophysica Acta* **1843**: 2685-2697

3.4 Investigation of the role of four mitotic septins and chitin synthase 2 for cytokinesis in *Kluyveromyces lactis*

D. Rippert & J. J. Heinisch,

(2016) *Fungal Genetics and Biology* **94:** 69-78

3.1 Mutations in SNF1 complex genes affect yeast cell wall strength

K. Backhaus, D. Rippert, C. J. Heilmann, A. G. Sorgo, C. G. de Koster, F. M. Klis, R. Rodicio & J. J. Heinisch, (2013)

European Journal of Cell Biology **92**: 383-395

Abstract

The trimeric SNF1 complex from *Saccharomyces cerevisiae*, a homolog of mammalian AMP-activated kinase, has been primarily implicated in signaling for the utilization of alternative carbon sources to glucose. We here find that *snf1* deletion mutants are hypersensitive to different cell wall stresses, such as the presence of Calcofluor white, Congo red, Zymolyase or the glucan synthase inhibitor Caspofungin in the growth medium. They also have a thinner cell wall. Caspofungin treatment triggers the phosphorylation of the catalytic Snf1 kinase subunit at Thr210 and removal of this phosphorylation site by mutagenesis (Snf1-T210A) abolishes the function of Snf1 in cell wall integrity. Deletion of the *PFK1* gene encoding the α-subunit of the heterooctameric yeast phosphofructokinase suppresses the cell wall phenotypes of a *snf1* deletion, which suggests a compensatory effect of central carbohydrate metabolism. Epistasis analyses with mutants in cell wall integrity (CWI) signaling confirm that the SNF1 complex and the CWI pathway independently affect yeast cell integrity.

DOI: 10.1016/j.ejcb.2014.01.001

3.2 Mig1 mediates regulation of cell wall synthesis by the SNF1 kinase complex in *Kluyveromyces lactis*

Running title: SNF1 and *K. lactis* cell wall

D. Rippert, K. Backhaus, S. Walter, R. Rodicio & J. J. Heinisch, (2016)

Department of Genetics, University of Osnabrück, Barbarastr.11,
49076 Osnabrück, Germany

manuscript in preparation

Abstract

The trimeric AMP-activated kinase complex (AMPK) is conserved from yeast to humans and is best known for its role in balancing energy metabolism. Additional functions, including the regulation of cell wall biosynthesis, have been proposed for the baker's yeast AMPK homologous SNF1 complex. We here demonstrate that this function is conserved in the Crabtree-negative milk yeast *Kluyveromyces lactis*. Deletion mutants in the genes encoding the subunits of the trimeric complex (*Klsnf1, Klgal83, Klsnf4*) displayed increased sensitivities towards cell wall stress agents and a mutant lacking the kinase subunit had a thinner cell wall in transmission electron micrographs as compared to wild type. Epistasis analyses demonstrated that the KlSNF1 complex acts in parallel to cell wall integrity (CWI) signaling and stress sensitivities of *Klsnf1* deletions can be suppressed by additional deletions in genes encoding enzymes of the upper part of glycolysis (*KlPFK1, KlPFK2, KlPGI1*) or the transcriptional repressor KlMig1. Western blot analyses of an HA-tagged KlMig1 revealed its phosphorylation on ethanol medium, but a substantial amount of protein remains phosphorylated even in the presence of high glucose concentrations. Application of cell wall stress shifted this equilibrium towards the non-phosphorylated fraction of KlMig1. Analyses using mass spectrometry showed that KlMig1 purified from cells grown on ethanol shared several conserved phosphoserines with the homologous residues in ScMig1, but also revealed residues differentially modified in the two yeast species. All data are consistent with the hypothesis that KlMig1 may exert a negative regulatory function on cell wall biosynthesis.

Introduction

The fungal cell wall is an elastic structure, which determines the shape of the cell and ensures cell integrity. Its synthesis is tightly regulated and approximately 1200 genes in *Saccharomyces cerevisiae* have been suggested to be involved in this process, either directly or indirectly (Klis *et al.*, 2002). Since mammalian cells are devoid of a cell wall and the walls of plant cells have a different composition, the fungal cell wall also provides an attractive target for the development of antifungal drugs (Heinisch, 2005). Electron micrographs generally show a bilayered structure of yeast cell walls, with an electron-transparent inner layer made up of ß-1,3- and ß-1,6-linked glucans and chitin, and an electron-dense outer layer composed of highly glycosylated mannoproteins. Synthesis and remodeling of the yeast cell wall at sites of lesions and polar growth are mediated by the cell wall integrity (CWI) signaling pathway (reviewed in Levin, 2005). Five sensor proteins located in the plasma membrane and penetrating the cell wall have been suggested to detect mechanical stress at the cell surface and relate the signal through the small GTPase Rho1 and the protein kinase C (Pkc1) to a conserved mitogen activated protein kinase cascade (MAPK; reviewed in Schmitz and Heinisch, 2003; Rodicio and Heinisch, 2010; Jendretzki *et al.*, 2011; Kock *et al.*, 2015). The downstream MAP kinase Slt2 finally phosphorylates the transcription factors Rlm1 and SBF. While the dimeric SBF complex, which consists of Swi4 and Swi6, relates cell wall synthesis to cell cycle control, Rlm1 activates at least 25 genes, whose products are involved in cell wall construction (Jung and Levin, 1999; Philip and Levin, 2001).

Recently, the SNF1 kinase complex was also found to affect cell wall synthesis in *S. cerevisiae* (Backhaus *et al.*, 2013). This complex is conserved throughout eukaryotes, belongs to the family of AMP-activated kinases (AMPKs), which are composed of three subunits, and generally senses and regulates the cells energy state (Hardie, 2003). In *S. cerevisiae* the heterotrimeric complex is composed of the catalytic α-subunit encoded by *SNF1*, either of three alternative ß-subunits, which determine subcellular localization and are encoded by *GAL83, SIP1* or *SIP2*, and the regulatory γ-subunit encoded by *SNF4* (Carlson *et al.*, 1981; Hedbacker and Carlson, 2008). The kinase activity is primarily involved in signaling the availability of hexose sugars to transcription factors such as Mig1, Cat8, Sip4 or Adr1. These control the expression of genes for the utilization of alternative carbon sources, such as sucrose, maltose or non-fermentable substrates (reviewed in Gancedo, 1998; Schüller, 2003). In addition, the yeast SNF1 complex affects a variety of cellular processes, including stress responses to high salt conditions, alkaline pH in the medium, oxidative stress, sporulation and aging (Honigberg and Lee, 1998; Ashrafi *et al.*, 2000; Portillo *et al.*,

2005; Hong and Carlson, 2007; Ye *et al.*, 2008). In addition, we found that mutations in *SNF1* or the genes encoding the other subunits generally display stress-sensitivities typical for mutants in the CWI pathway and cause a pronounced decrease in cell wall thickness (Backhaus *et al.*, 2013). Whether these effects are mediated by the prominent downstream transcription factor Mig1 remained unclear. In glucose signaling, Mig1 is a transcriptional repressor for genes involved in the utilization of alternative carbon sources (Nehlin *et al.*, 1991). It has been proposed that upon glucose depletion the SNF1 kinase complex becomes activated and phosphorylates Mig1, which then shuttles to the cytoplasm and ceases to repress nuclear gene expression (DeVit *et al.*, 1997; Östling and Ronne, 1998; Treitel *et al.*, 1998). This model has been refined recently by the demonstration that a substantial portion of Mig1 remains in the nucleus even at low glucose concentration, but is part of a large repressor complex, which only acts on gene expression when glucose is abundant (Vega *et al.*, 2016).

With the development of powerful molecular genetic tools and its much lower degree of genetic redundancy the milk yeast *Kluyveromyces lactis* has become an alternative model to study basic cellular processes (Rodicio and Heinisch, 2013). Although its cell wall is approximately 40% thinner than the one of *S. cerevisiae* (Backhaus *et al.*, 2010), the CWI pathway is similarly structured. Yet, the reduced genetic redundancy of *K. lactis* is reflected by the fact that only three instead of five genes encode CWI sensors (Rodicio *et al.*, 2008) and that only one isozyme exists for KlRom2 and KlMkk1 (reviewed in Backhaus *et al.*, 2011).

K. lactis is a Crabtree-negative yeast with a predominantly respiratory metabolism and a lower degree of glucose repression, e.g. for mitochondrial functions (Tarrío *et al.*, 2006). Glucose repression, although less stringent than in *S. cerevisiae*, is mediated by the orthologous SNF1 kinase complex. In *K. lactis* the latter is composed of the α-subunit KlSnf1 (alias Fog2, for fermentative and oxidative growth), a single ß-subunit KlGal83 (alias Fog1), and the putative γ-subunit KlSnf4, as deduced from the identification of a *ScSNF4* homolog in the *K. lactis* genome and reported herein (Goffrini *et al.*, 1996; Dong and Dickson, 1997; http://genome.jgi.doe.gov/Klula1/Klula1.home.html). Orthologs of the major effector proteins, such as KlMig1, KlCat8 and KlSip4 have also been described, with notable differences in their target range as compared to *S. cerevisiae* (Dong and Dickson, 1997; Lodi *et al.*, 2001). For example, KlMig1 represses *KlGAL1* transcription but does not impair expression of the invertase gene, and KlSip4 plays a role in assimilation of C2 carbon compounds, which is not found in *S. cerevisiae* (Mehlgarten *et al.*, 2015). Moreover, whether the hexokinase PII function in glucose repression, which was observed in *S. cerevisiae* and attributed to its interaction with Mig1 in the nucleus (Ahuatzi *et al.*, 2007), is shared by the sole hexokinase

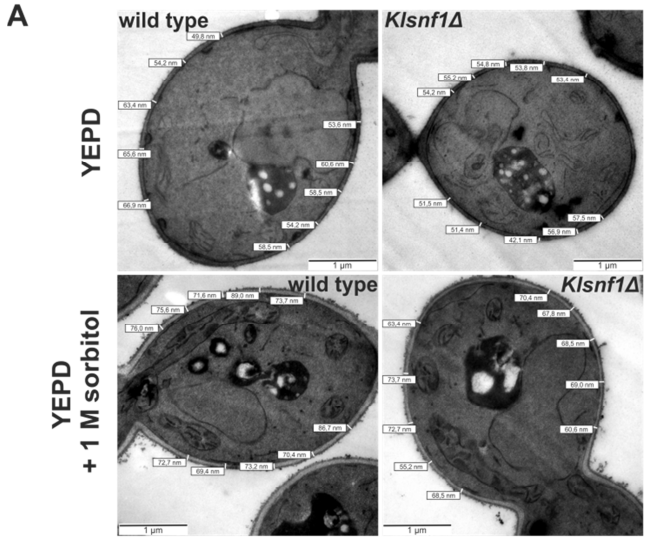
of *K. lactis* encoded by the *RAG5/KlHXK1* gene is still a matter of debate: Whereas in heterologous complementation studies only its sugar phosphorylating activity appeared to be conserved (Prior *et al.*, 1993), a recent work performed with *Klhxk1* deletion mutants, in a different *K. lactis* strain more susceptible to glucose repression, suggests its involvement not only in glucose sensing, but also in general regulatory processes (Mates *et al.*, 2014).
We here found that *K. lactis* mutants with defects in the SNF1 complex have thinner cell walls than their congenic wild type and are hypersensitive to cell wall stress. Unlike the differences in control of carbon source utilization, these aspects of SNF1 function are shared with its *S. cerevisiae* ortholog. We also found that the downstream transcription factor KlMig1 is involved in mediating the cell wall integrity response.

Results

The SNF1 complex is required for proper cell wall construction

We previously observed that cell wall thickness in *S. cerevisiae* is reduced by approximately 30% in mutants defective in the SNF1 complex as compared to wild type (Backhaus *et al.*, 2013). To test whether this is also true in *K. lactis*, we first examined the ultrastructure of the cell wall in a *Klsnf1* deletion mutant constructed for this purpose in a congenic strain background (Fig. 1A). Two strains carrying this deletion were first grown in rich medium and the cell wall thickness was compared to that of a *KlSNF1* wild-type strain. No clear differences could be observed in measurements of transmission electron microscopy (TEM) images, with one mutant giving the same values as the wild type and the other one having only a slightly thinner cell wall. We reasoned that due to the generally thinner cell wall of *K. lactis* as compared to *S. cerevisiae*, mutants further decreasing this thickness may be prone to lysis either during growth and/or in the preparation for TEM and thus be lost prior to image generation, leaving only a population of thicker-walled cells. Therefore, cells were grown in the presence of 1 M sorbitol for osmotic stabilization and cell wall thickness was again determined. As evident from the data in Fig. 1A these growth conditions resulted in an approximately 50% increase in cell wall thickness in wild-type cells. However, compared to the latter, the cell walls in *Klsnf1* mutants were approximately 15% thinner.
In order to establish a less laborious method to assess the effect of mutations on cell wall integrity, we performed drop dilution assays on media with different cell wall stress agents, using growth on ethanol medium as a control for KlSNF1 function (Fig. 1B). For this purpose, deletion mutants were additionally constructed in the genes encoding the only ß-subunit KlGal83 and the γ-subunit KlSnf4. As expected, the three single deletion mutants all lacked the ability to grow on ethanol, indicating a defect in derepression of gluconeogenic genes

(Fig. 1B). *Klsnf1*Δ and *Klsnf4*Δ were also sensitive to cell wall stress, shown by their impaired growth on Caspofungin, Calcofluor white and Congo red. *Klgal83*Δ only showed a growth inhibition on Calcofluor white but not in the presence of the other two stress agents in the concentrations tested. We concluded that a functional KlSNF1 complex is required for proper cell wall construction in *K. lactis*. Since these phenotypes are reminiscent of our findings for *Scsnf1*Δ mutants (Backhaus *et al.*, 2013), we assessed the ability of *ScSNF1* to rescue the *Klsnf1*Δ phenotypes and found that both the defects in carbon-source derepression and in growth under cell wall stress can be complemented by the heterologous gene (Fig. S1).



Strain	Cell wall thickness [nm]	
	YEPD	YEPD + 1 M sorbitol
wild type	59.7 ± 6.9	89.5 ± 13.6
Klsnf1Δ	59.6 ± 6.5	78.0 ± 11.0
Klsnf1Δ	54.3 ± 4.5	73.9 ± 8.5

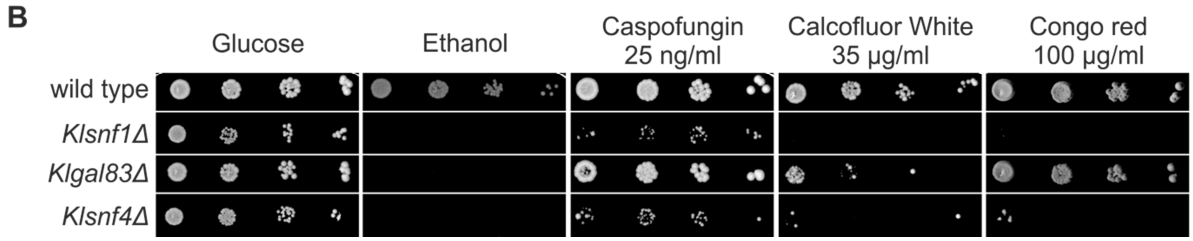


Figure 1: Phenotypic characterization of mutants of the KlSNF1 complex. A) Cell wall thickness is reduced in *Klsnf1* mutants. Cells growing logarithmically in rich medium with or without 1 M sorbitol were fixed for transmission electron microscopy and cell wall thickness was determined at the points indicated. Values represent the average of the walls of 40 cells measured at 10 different points, each. B) Mutants lacking any of the three KlSNF1 subunits are hypersensitive to cell wall stress. Cells growing exponentially in rich medium were adjusted to an OD_{600} of 0.1. Dilutions from 10^0 to 10^{-3} were spotted with 3 µl each onto synthetic media containing the indicated stress agents and incubated at 30℃ f or 3 days.

The SNF1 complex and CWI signaling act in parallel on cell wall synthesis

Cell wall synthesis in yeast is controlled by the CWI signaling pathway, which is basically conserved between *S. cerevisiae* and *K. lactis* (Backhaus *et al.*, 2011). Thus, *Klbck1*Δ and *Klmpk1*Δ strains show an osmo-remedial lysis phenotype (Jacoby *et al.*, 1999; Kirchrath *et al.*, 2000). We here constructed new deletion mutants in these two genes in the congenic

background, as well as a deletion in the gene encoding the transcriptional activator KlRlm1. As shown in Fig. 2, single mutants were able to grow on synthetic medium without osmotic stabilization at 25℃ and at low concentrations of cell wall stressors, as was the *Klsnf1*Δ mutant. However, each of the CWI mutants failed to grow without osmotic stabilization when combined with the *Klsnf1*Δ allele. This synthetic growth defect was rescued by the addition of 1 M sorbitol. The additive phenotypes indicate that the signaling pathways controlled by the SNF1 complex and the CWI cascade act in parallel on cell wall synthesis, consistent with our observations in *S. cerevisiae* (Backhaus *et al.*, 2013).

A block in glycolysis rescues the cell wall defects of a *Klsnf1* null mutant

In *S. cerevisiae* the cell wall defects caused by a *Scsnf1* deletion could be partially suppressed by a concomitant deletion of the *ScPFK1* gene encoding the α-subunit of the heterooctameric phosphofructokinase (Backhaus *et al.*, 2013). This was attributed to an intracellular accumulation of glucose-6-phosphate, which is a major precursor of cell wall polysaccharides. However, other glycolytic mutants which would also accumulate glucose-6-phosphate could not be tested in *S. cerevisiae*, since they do not grow on glucose (Ciriacy and Breitenbach, 1979). By contrast, a block in glycolysis can be bypassed in *K. lactis* by the pentose phosphate pathway and allows such mutants to grow (Jacoby *et al.*, 1993). We therefore constructed deletion mutants in the congenic strain background in the genes encoding either subunit of phosphofructokinase (*Klpfk1*Δ and *Klpfk2*Δ; Heinisch *et al.*, 1993), as well as in the gene for phosphoglucose isomerase (*Klpgi1*Δ; Goffrini *et al.*, 1991). None of the single deletions affected growth on glucose media in the presence of cell wall stress agents compared to wild-type or non-stress controls (Fig. 3A). Yet, the growth defect of the *Klsnf1*Δ mutant on Caspofungin, Calcofluor white and Congo red was suppressed when either of the glycolytic null alleles was introduced. We proceeded to measure the intracellular concentrations of the sugar phosphates as precursors of cell wall synthesis in cell extracts. Metabolite concentrations were determined in wild-type cells and all possible mutant combinations after growth on rich medium with 4% glucose. As evident from Fig. 3B, glucose-6-phosphate levels were reduced approximately by half in the *Klsnf1*Δ strain, but exceeded wild-type levels in the different double mutants, i.e. *Klsnf1*Δ *Klpgi1*Δ (≈100-fold), *Klsnf1*Δ *Klpfk1*Δ (≈2-fold), and *Klsnf1*Δ *Klpfk2*Δ (≈20-fold). Interestingly, the concentrations of fructose-6-phosphate and fructose-1,6-bisphosphate in the *Klsnf1*Δ mutant were similar or reduced as compared to the wild type (no change and ≈0.5-fold, respectively), but generally higher than wild type in all glycolytic mutants and the double mutants. Regarding the suppression of cell wall defects caused by *Klsnf1*Δ, these data are consistent with the

hypothesis that accumulation of the precursor glucose-6-phosphate can provide a mechanism to enforce cell wall synthesis.

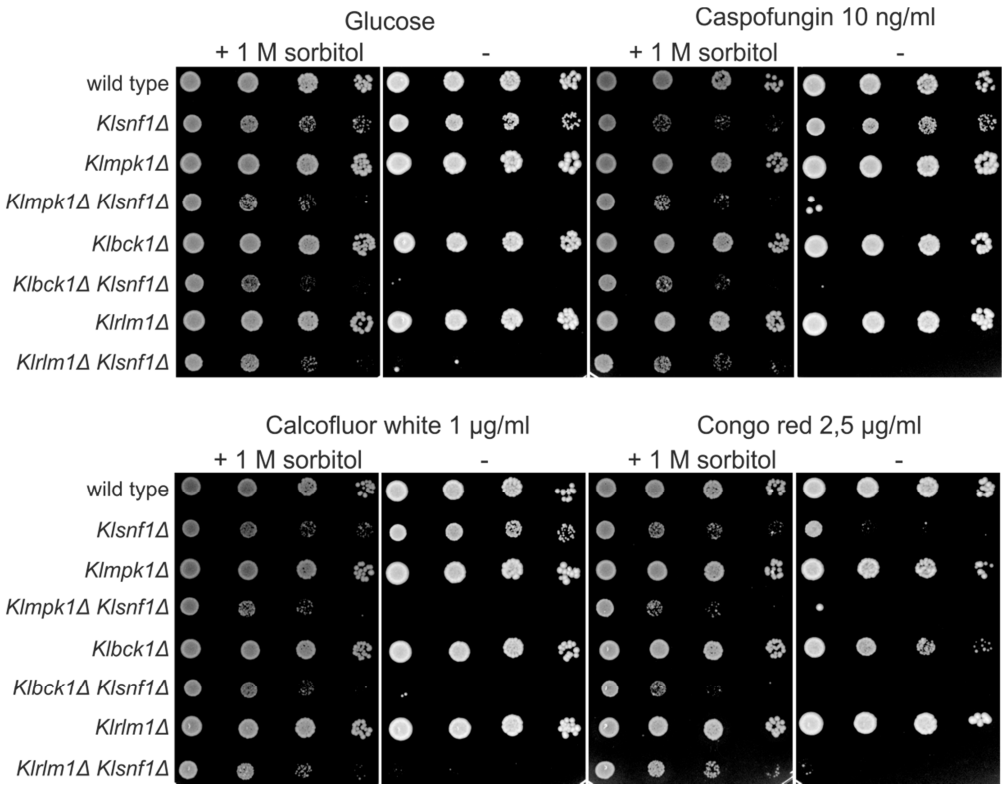


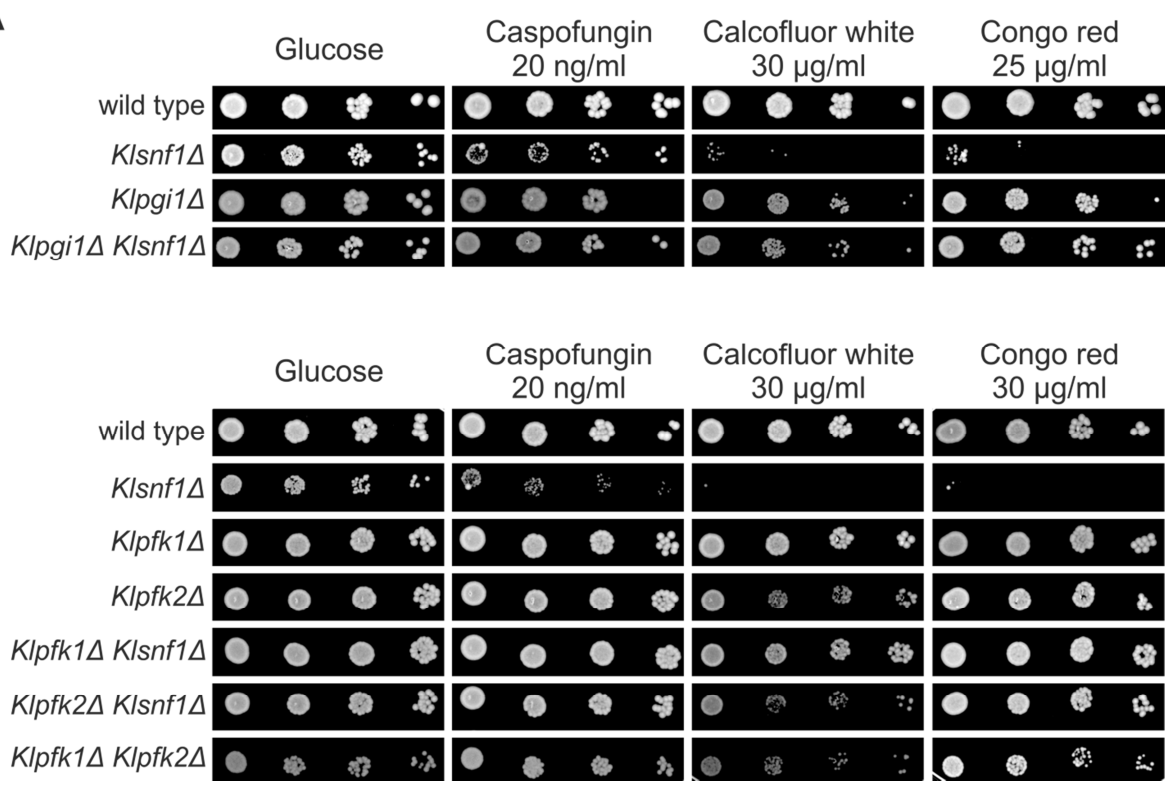
Figure 2: Epistasis analyses of KlSNF1 and CWI signaling mutants. Growth of the single and double deletions in KlSNF1 and CWI pathway genes as indicated was tested. Cells growing exponentially in rich medium containing 1 M sorbitol were adjusted to an OD_{600} of 0.1. Dilutions from 10^{0} to 10^{-3} were spotted onto synthetic media containing the indicated stress agents and incubated at 25℃ for 3 days.

The Snf1 target transcription factor KlMig1 is involved in the control of cell wall synthesis

KlMig1 was identified as a functional ortholog of a transcriptional repressor and target of Snf1 in *S. cerevisiae* (Cassart *et al.*, 1995). In *K. lactis* it is also involved in glucose repression, for instance it regulates lactose metabolism, but not invertase gene expression (Dong and Dickson, 1997; Georis *et al.*, 1999). We here tried to determine whether or not KlMig1 mediates the cell wall response triggered by the KlSNF1 complex. For this purpose, a deletion of the *KlMIG1* open reading frame was obtained in the congenic strain series and first employed in epistasis analyses. The drop dilution assays presented in Fig. 4A show that the sensitivities of a *Klmig1*Δ mutant towards cell wall stress agents were similar to wild type. However, the growth defects of the *Klsnf1*Δ strain on Caspofungin, Calcofluor white and Congo red were suppressed in the *Klsnf1*Δ *Klmig1*Δ double mutant. This indicates that KlMig1 exerts a negative regulatory function on cell wall synthesis, which is abrogated in the

null mutant. As expected, growth on ethanol as a carbon source was not restored in the double deletion, consistent with its role in glucose signaling.

A



B

Strain	Relevant genotype	Glucose-6-phosphate (nmoles/mg dw)	Fructose-6-phosphate (nmoles/mg dw)	Fructose-1,6-bisphosphate (nmoles/mg dw)
KDR1-9B	wild type	0.27 ± 0.17	0.16 ± 0.05	2.19 ± 1.28
KDR1-1C	*Klsnf1*Δ	0.15 ± 0.13	0.17 ± 0.08	0.94 ± 0.60
KDR50-4C	*Klpgi1*Δ	25.85 ± 5.72	0.71 ± 0.07	1.16 ± 0.16
KDR57-6A	*Klpgi1*Δ *Klsnf1*Δ	24.79 ± 2.60	0.97 ± 0.15	2.47 ± 0.05
KDR22-8A	*Klpfk1*Δ	1.68 ± 0.46	0.76 ± 0.34	5.06 ± 1.35
KDR22-1C	*Klpfk1*Δ *Klsnf1*Δ	0.63 ± 0.08	0.99 ± 0.08	3.71 ± 0.06
KDR24-7A	*Klpfk2*Δ	3.08 ± 0.69	1.56 ± 0.27	3.38 ± 1.12
KDR24-1C	*Klpfk2*Δ *Klsnf1*Δ	5.25 ± 0.11	2.96 ± 0.20	4.62 ± 1.28

Figure 3: Effect of glycolytic mutants on SNF1-mediated cell wall synthesis. A) Additional deletions of *KlPFK1*, *KlPFK2* or *KlPGI1* reduce the cell wall defects of *Klsnf1* deletion strains. Drop dilution assays were performed as described in the legend of Fig. 1. B) Metabolite determinations show accumulation of sugar phosphates. Levels of the indicated sugar phosphates were enzymatically determined in wild-type cells and different deletion strains. Metabolite extracts were prepared from 100 ml cultures in YEPD (4% glucose) and treated as described in the experimental procedures. The average of two independent biological replicates are given.

Based on sequence alignments and data obtained from expression of *KlMIG1* variants in *S. cerevisiae* it has been suggested that the protein is phosphorylated at conserved serine residues upon glucose depletion (DeVit and Johnston, 1999). However, to our knowledg

phosphorylation of KlMig1 in its native environment has not yet been demonstrated. In order to look for the effects of cell wall stress on this posttranslational modification, we constructed a KlMig1-3xHA-tagged variant and performed Western analyses on extracts from cells grown under different conditions (Fig. 4B). Wild-type cells grown on ethanol and a *Klsnf1*Δ deletion grown either on glucose or on ethanol served as controls and showed that a faster migrating band corresponds to the non-phosphorylated form of KlMig1 and several slower migrating bands where therefore assumed to represent phosphorylated derivatives. Approximately two-thirds of KlMig1 appeared to be phosphorylated even when grown on glucose, and only one third was not modified (Fig. 4B). This relation of phosphorylated versus unphosphorylated KlMig1 was reversed after treatment of *K. lactis* cells for 40 min with Caspofungin. Calcofluor white treatment showed a similar, albeit weaker tendency for increasing the relative amount of unphosphorylated KlMig1. Thus the concentration of KlMig1 in the nucleus is expected to increase under cell wall stress, presumably exerting its repressor function on cell wall biosynthetic genes indicated by the epistasis analyses provided above.

The localization of the repressor in life cells was tested using a KlMig1-GFP fusion, which was introduced into a *Klmig1*Δ strain encoded by a plasmid-born copy (Fig. 4C). Consistent with the data of the Western blot, KlMig1-GFP was detected in the nucleus and the cytoplasm even when grown in high glucose concentrations (2%), but was almost exclusively cytoplasmic when cells were shifted for 1 h to ethanol medium. Attempts to follow KlMig1-GFP localization upon addition of Caspofungin as a cell wall stress agent did not yield reproducible results, in part attributed to the fact that the GFP signal was undetectable after Caspofungin treatment (data not shown).

The Mig1 function in cell wall synthesis is conserved in *S. cerevisiae*

The role of Mig1 in cell wall related SNF1 signaling in *S. cerevisiae* has not been investigated, yet. Given the high degree of similarity of the Mig1 orthologs (DeVit and Johnston, 1999), we therefore decided to test, whether a *Scmig1* deletion can also suppress the phenotypes of a *Scsnf1* null mutant. Drop dilution assays on different stress media confirmed, that the hyper-sensitivities of *Scsnf1* towards Caspofungin, Calcofluor white and Congo red were also alleviated by a concomitant *Scmig1* deletion (Fig. 5A). Assessment of the ScMig1 phosphorylation status under various growth conditions revealed several differences to its ortholog in *K. lactis* (Fig. 5B). Although it is completely phosphorylated on ethanol medium in wild-type and not at all phosphorylated in the *Scsnf1* deletion on this medium, as expected, glucose-grown cells yield a number of bands with retarded mobility, moving in between the unphosphorylated and the completely phosphorylated controls. The appearance of these intermediate bands in the glucose-grown *Scsnf1* deletion indicates that

they are due to modifications of ScMig1 independent of the Snf1 kinase activity. By contrast to *K. lactis* only a very weak band could be detected at the position of fully phosphorylated ScMig1 in glucose-grown cells, indicating that the vast majority of the protein may reside in the nucleus. This effect appeared to be enhanced under cell wall stress, as the faint fully phosphorylated band approached the level of detection. Since the nuclear export machinery of *S. cerevisiae* also works on KlMig1 (DeVit and Johnston, 1999), we decided to test the localization of KlMig1-GFP in this yeast. As shown in Fig. 5C and consistent with the data of immunodetection, the repressor was confined to the nucleus in glucose-grown cells, independent of the presence of cell wall stress agents. On the other hand, it appeared almost exclusively cytoplasmic after a shift for 1 h to ethanol medium.

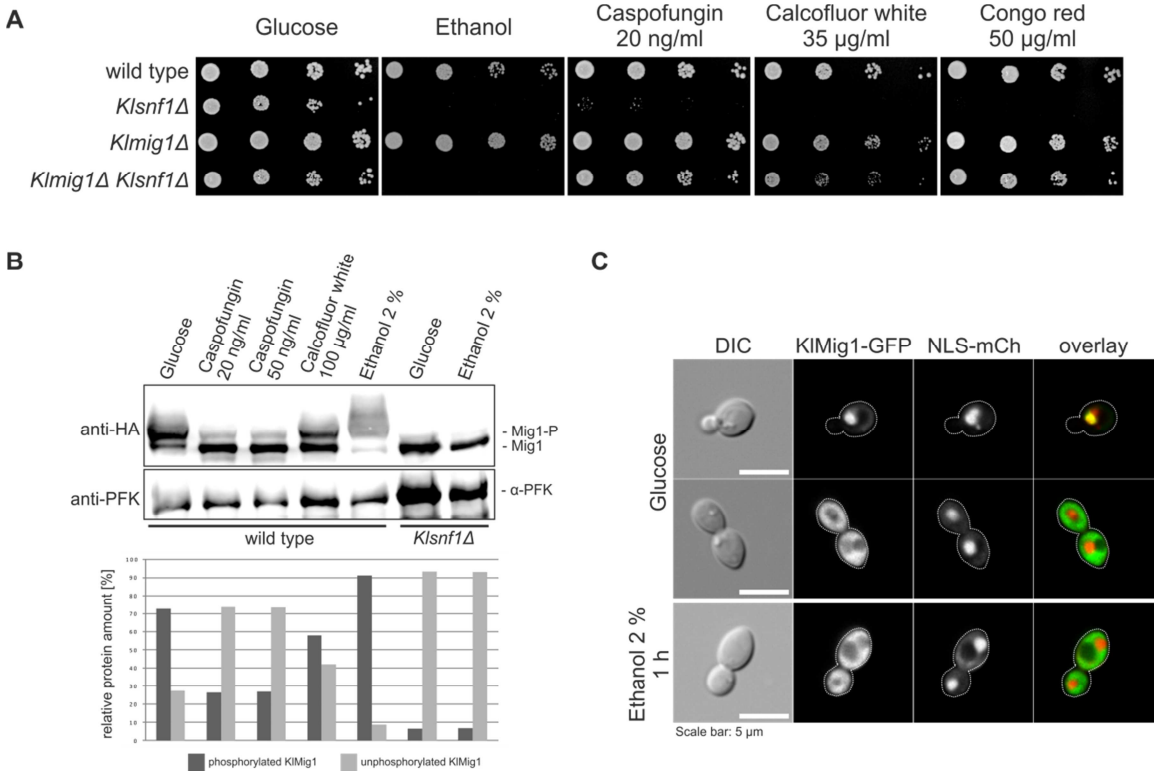


Figure 4: The role of KlMig1 in the control of cell wall synthesis. A) Epistasis analyses of *Klsnf1* and *Klmig1* deletion strains. Drop dilution assays as indicated were performed as described in the legend of Fig. 1. B) Western blot analysis to assess the phosphorylation state of KlMig1. Wild-type cells and *Klsnf1* mutants carrying a genomically tagged KlMig1-3xHA were grown in rich medium (containing 2% glucose for wild-type cells and 4% glucose for *Klsnf1* mutants) to an OD_{600} of 1. As a control for maximal phosphorylation, a sample was grown on ethanol as a carbon source. Caspofungin was added to a final concentration of 50 ng/ml and Calcofluor white was added to a final concentration of 100 µg/ml. Immunological detection was performed as detailed in the experimental procedures. C) Localization of GFP-tagged KlMig1 in life cells. A *KlMIG1-GFP* fusion was introduced on a multicopy plasmid into a genomic *Klmig1* deletion and cells were grown on selective medium for plasmid maintenance and employed for localization studies after growth on glucose or ethanol.

The phosphorylation sites in KlMig1 are partially conserved as compared to ScMig1

To shed some light on the regulation of KlMig1 we decided to investigate the role of conserved serine residues which have been shown to be phosphorylated by the Snf1 kinase

in ScMig1. For this purpose, we first exchanged a total of seven serine residues at conserved sites for alanines as shown in Fig. 6A. GFP fusions of such mutants demonstrated that in contrast to the wild-type they at least partially remained in the nucleus even under derepressing conditions on ethanol medium in *K. lactis* (Fig. 6B).

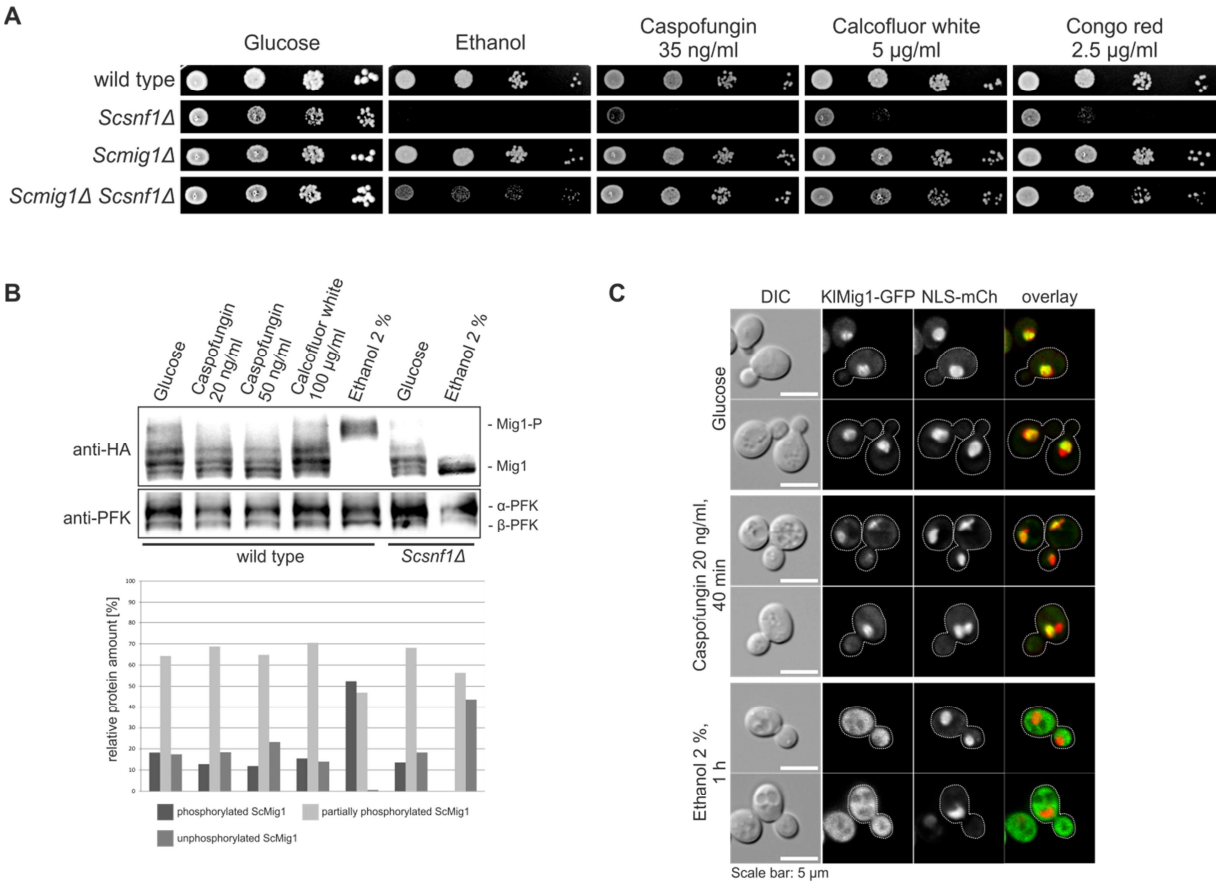


Figure 5: Role of ScMig1 in cell wall synthesis in *S. cerevisiae*. A) Phenotypes of single and double mutants lacking *ScSNF1* and *ScMIG1* were tested in a drop dilution assay as described in the legend of Fig. 1. B) Western blot analyses to determine the phosphorylation state of ScMig1. Preparation and immunological detection was performed as described in the legend of Fig. 4B. C) Localization studies of a plasmid-borne KlMig1-GFP in *S. cerevisiae*. KlMig1-GFP was introduced into a *Scmig1* deletion strain and cells were grown in selective medium for plasmid maintenance, supplemented with the carbon sources indicated and after addition of 20 ng/ml Caspofungin, where indicated.

In order to determine which residues are in fact phosphorylated in KlMig1 *in vivo*, the coding sequence for KlMig1 was fused to that of a TAP-Tag and the construct was introduced on a multicopy vector into a *Klmig1* deletion strain. Transformants were grown on selective medium with ethanol as a carbon source and used to purify the tagged protein. A subsequent analysis by mass-spectrometry revealed a peptide coverage of only 37%. Nevertheless, Ser329, which is equivalent to Ser311 in ScMig1 important for nuclear export (Ahuatzi *et al.*, 2007), was clearly not phosphorylated in the *K. lactis* homolog (Fig. 6C, Table S2). On the other hand, we detected a phosphorylation at Ser345 (approximately 47% of these residues

in the purified protein prepared from cells grown on ethanol), a residue which does not conform to the consensus sequence for a ScSnf1 target and is equivalent to the non-phosphorylated Ser328 in ScMig1. The mass-spectrometry data of the peptide containing Ser345 is shown in Fig. S2. Although this indicates subtle differences in the regulation of the shuttle mechanism between nucleus and cytoplasm for Mig1 in the two yeast species, in the light of the complementation studies cited above we believe that the general regulatory principles are conserved. Experiments to increase the peptide coverage and to determine the phosphorylation pattern in response to addition of glucose and/or Caspofungin are currently in progress.

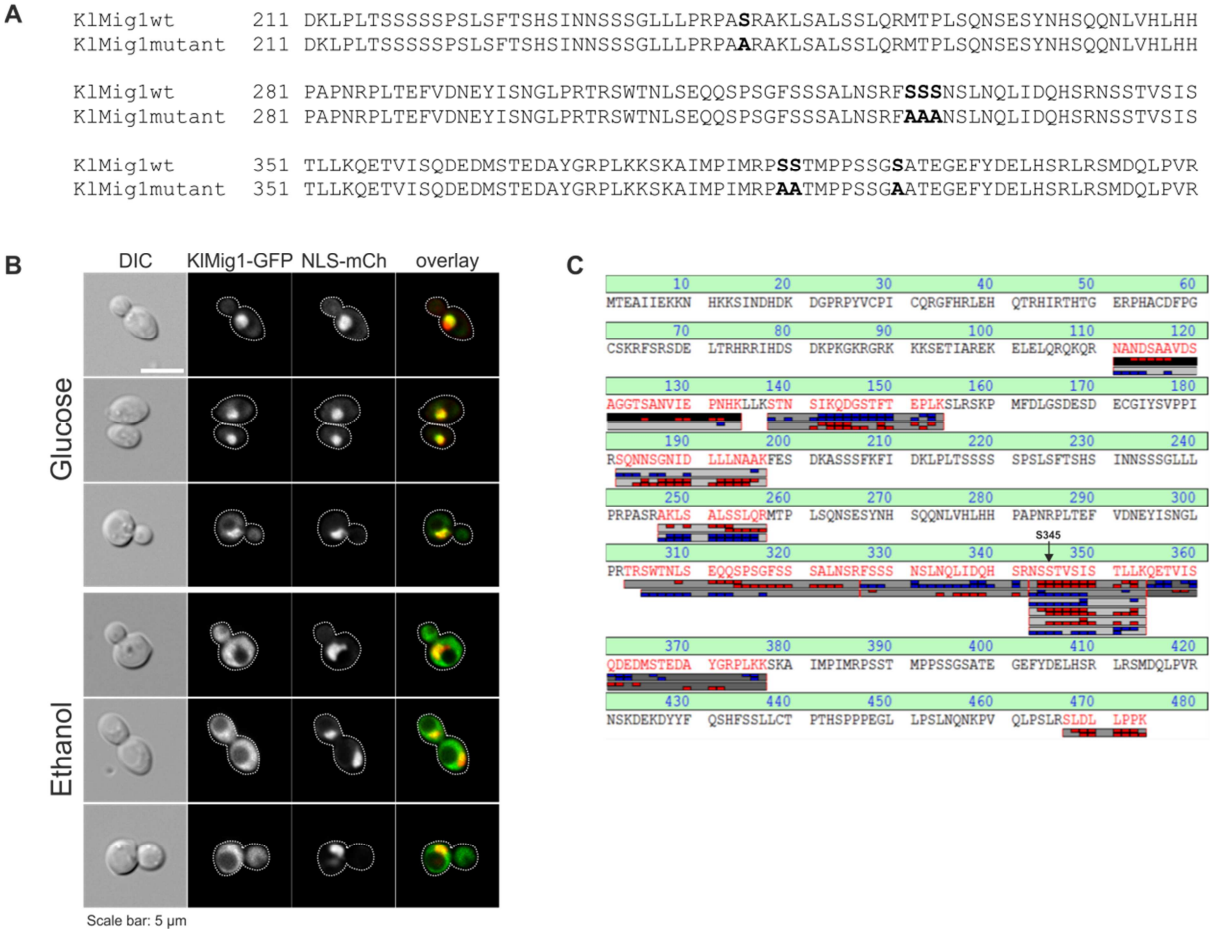


Figure 6: Determination of phosphorylation sites in KlMig1. A) Sequence alignment of a wild-type KlMig1 and a mutant of KlMig1 with seven exchanges of serines to alanines at putative KlSnf1 phosphorylation sites. B) Intracellular localization of the phosphorylation-impaired KlMig1 mutants. A plasmid-borne copy of the KlMig1 mutant was introduced into a *Klmig1* deletion strain and localization was examined after growth on synthetic medium containing either glucose or ethanol as carbon sources. C) Mass-spectrometry analysis of purified KlMig1. The sequence of KlMig1 and peptide coverage are shown. Blue bars indicate the EDT data and red bars indicate the CID data. The phosphorylated serine residue is marked with an arrow.

Discussion

A primary function of the trimeric yeast SNF1 complex, and its AMPK orthologs in higher eukaryotes, is the balancing of energy metabolism through phosphorylation of downstream effectors, which trigger the proper cellular responses towards changing nutrient conditions (Hedbacker and Carlson, 2008). Yet, the downstream transcriptional network mediating this response already diverged between the two yeast species *S. cerevisiae* and *K. lactis* (Mehlgarten *et al.*, 2015). We recently discovered an additional function of the SNF1 complex in *S. cerevisiae* related to cell wall integrity, but the downstream effectors for this function remained obscure (Backhaus *et al.*, 2013).

In the present work we therefore commenced by confirming the cell wall-related function of the SNF1 complex in the genomically less redundant yeast *K. lactis* (Rodicio and Heinisch, 2013). Besides the characterization of KlSnf4 as a putative regulatory subunit, our data from TEM measurements of cell wall thickness and the assessment of sensitivities of deletion mutants lacking one of the three subunits towards cell wall stress agents basically confirmed the involvement of the KlSNF1 complex in the maintenance of cell integrity found in *S. cerevisiae* (Backhaus *et al.*, 2013). This notion was supported by the results of our epistasis analyses, demonstrating the KlSNF1 signaling works in parallel to CWI signaling in *K. lactis*. A notable difference to *S. cerevisiae* were the comparatively moderate sensitivities of a *Klgal83* deletion towards cell wall stress agents. The three alternative ß-subunits in *S. cerevisiae* have been proposed to govern subcellular localization, with ScGal83 necessary to direct the SNF1 complex to the nucleus (Vincent *et al.*, 2001; Hedbacker and Carlson, 2006). Since KlGal83 is the only ß-subunit available to *K. lactis*, we propose that its primary function is not involved in directing the subcellular localization of the complex. Thus, KlSnf1 kinase subunits reaching the nucleus may be sufficient to partially fulfill their function in regulating cell wall synthesis even in the absence of KlGal83. The primary role of the Snf1 subunit is further underlined by the observation, that the *ScSNF1* gene can fully complement all the defects in carbon-source response and cell wall biosynthesis observed in a *Klsnf1* deletion. Thus, the structure of ScSnf1 is sufficiently conserved to substitute its *K. lactis* ortholog in a heterotrimeric complex, in contrast to expression of a gene encoding a human kinase subunit in a *Scsnf1* deletion (Ye *et al.*, 2014).

Our epistasis analyses not only indicated that the KlSNF1 complex acts in parallel to CWI signaling, but also that mutations in genes encoding glycolytic enzymes can suppress the hyper-sensitivity of *Klsnf1* deletions towards cell wall stress agents, consistent with data reported on *Scsnf1 Scpfk1* double mutants (Backhaus *et al.*, 2013). Since in the latter work it has been proposed that accumulation of glucose-6-phosphate as a precursor for synthesis of

cell wall polysaccharides could be responsible for this suppression phenotype, we here took advantage of the fact that, in contrast to *S. cerevisiae*, *K. lactis* can grow on glucose in the absence of glycolysis (Goffrini *et al.*, 1991; Heinisch *et al.*, 1993), which has been attributed to a high capacity of the pentose phosphate pathway (PPP) in milk yeast (Jacoby *et al.*, 1993; Saliola *et al.*, 2007; Merico *et al.*, 2009). Nevertheless, glycolytic mutants were expected to accumulate the substrate sugar phosphates feeding the respective reactions, as shown in *S. cerevisiae* (Ciriacy and Breitenbach, 1979). In line with the notion that the accumulation of cell wall polysaccharide precursors can overcome the cell wall-related phenotypes of a *Klsnf1* mutant, we found that additional *Klpgi1/rag2, Klpfk1* or *Klpfk2* deletions indeed suppressed the growth defects under cell wall stress. A reduced concentration of glucose-6-phosphate and fructose-6-phosphate was indeed determined in metabolite extracts from *Klsnf1* mutants and shown to dramatically increase in the strains carrying any of the glycolytic gene deletions and in the double mutants with *Klsnf1*. Besides supporting the important role of sugar metabolites for cell wall synthesis, these data, obtained for the first time in glycolytic mutants of *K. lactis* to our knowledge, are also interesting in a broader physiological sense: At first sight, one would expect the observed accumulation of substrates in the glycolytic mutants, but also a decrease in the product concentrations, i.e. fructose-1,6-bisphosphate levels should decline in the *Klpfk1* and *Klpfk2* mutants, as in addition should the level of fructose-6-phosphate in the *Klpgi1* mutant. We attribute the paradoxical accumulation of these sugar phosphates in the mutants to the interconnections between glycolysis and the PPP, as depicted in Fig. 7. An increased flow of glucose-6-phosphate into the PPP caused by the block in glycolysis, may first of all lead to a metabolic imbalance at the level of the glucose-6-phosphate dehydrogenase (KlZwf1) reaction (Saliola *et al.*, 2007; Lamas-Maceiras *et al.*, 2015). Moreover, the sugar phosphates can be replenished by a backflow from the PPP into glycolysis at the levels of fructose-6-phosphate and glyceraldehyde-3-phosphate (GAP; Heinisch and Zimmerman, 1985). Thus, our data support the previously assumed high capacity of the PPP in *K. lactis* as reflected by sugar metabolite concentrations.

Most importantly, the downstream effectors of the SNF1 complex in yeast related to the cell wall functions remained elusive, until now. Both the capacity of a *mig1* deletion to suppress *snf1* cell wall-related phenotypes in either *S. cerevisiae* or *K. lactis*, as well as the observation that cell wall stress agents such as Caspofungin decrease the phosphorylation level of the Mig1 repressor, argue for its role as a SNF1 effector also with respect to cell wall synthesis.

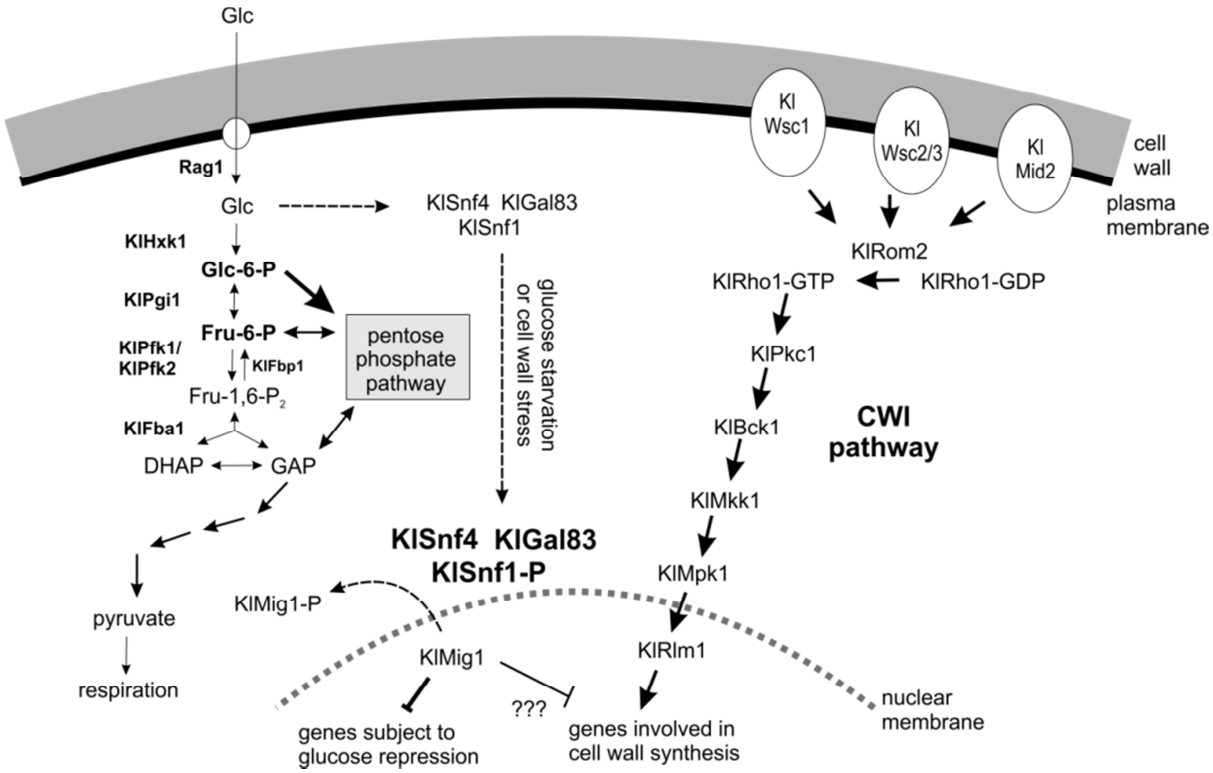


Figure 7: Presumed model of KlSNF1 and KlMig1 interaction. Glc=Glucose, Glc-6-P=Glucose-6-phosphate, Fru-6-P=Fructose-6-phosphate, Fru-1,6-P_2=Fructose-1,6-bisphosphate, DHAP=Dihydroxyacetonphosphate, GAP=Glyceraldehyde-3-phosphate, Rag1=Low-affinity glucose transporter, KlHxk1=Hexokinase, KlPgi1=Phophoglucose isomerase, KlPfk1/KlPfk2=Phosphofructokinases, KlFbp1=Fructose-1,6-bisphosphatase, KlFba1=Fructose-1,6-bisphosphate aldolase, KlSnf1/KlSnf4/KlGal83=KlSNF1 complex, KlMig1=Transcription factor; KlWsc1/KlWsc2/3/KlMid2=cell wall sensors, KlRho1=GTP-binding protein; GTP=Guanosin triphosphate, GDP=Guanosine diphosphate, KlRom2=Guanine nucleotide exchange factor (GEF), KlPck1=Protein kinase C, KlBck1=Mitogen-activated protein (MAP) kinase kinase kinase, KlMkk1=MAP kinase kinase, KlMpk1=MAP kinase, KlRlm1=MADS box transcription factor, ?=possible connection.

The suppression phenotype further indicates that it should function as a negative regulator, consistent with its role in glucose signaling. Yet, the exact mechanism of Mig1 function in regulating cell wall synthesis is a matter of speculation. One possibility is depicted in Fig. 7. As outlined above, sugar phosphates are an energy source and serve as precursors for the synthesis of cell wall polysaccharides. Mig1 may thus act in balancing these pathways under cell wall stress: while activating the CWI pathway, dephosphorylation of Mig1 as supported by our data would increase its concentration, and presumably its inhibitory activity on expression of cell wall biosynthetic genes. Although one may therefore expect a *mig1* deletion to cause a hyper-resistance to cell wall stressors, its apparent absence would be explained by the counteracting effect of energy wasted in the synthesis of access cell wall polysaccharides.

In this scenario, our finding that despite the conservation of key phosphorylation sites in Mig1 of *S. cerevisiae* and *K. lactis*, certain serine residues are exclusively modified in one or the other yeast, may reflect different degrees of modulation of their subcellular localization in response to carbon sources and cell wall stress, as found in our life-cell images.

In conclusion, our findings clearly support a conserved role of the SNF1 complex and its downstream effector Mig1 in the regulation of yeast cell wall biosynthesis. Although still highly speculative, the proposed interconnection between energy metabolism, pentose phosphate pathway and CWI signaling opens up an entire new field of research.

Experimental procedures

Strains, genetic manipulations, media and culture conditions

Genotypes of *K. lactis* and *S. cerevisiae* strains employed in this work are listed in Table 1. For purposes of homologous recombination in *S. cerevisiae* the strain DHD5 (*MATa/MATalpha ura3-52/ura3-52 his3-11,15/his3-11,15 leu2-3,112/leu2-3,112*; Kirchrath *et al.*, 2000) was employed. Otherwise, the BY4743 strain background from the deletion mutant collection was employed, using either deletions from the collection (substitutions by the *kanMX* marker) or generating some of the deletions with auxotrophic markers as required (Brachmann *et al.*, 1998). For plasmid amplification in *E. coli*, strain DH5α was used (Invitrogen, Karlsruhe, Germany). Standard procedures were followed for genetic manipulations of yeasts and plasmid constructions, as described (Rodicio *et al.*, 2008). A list of the plasmids employed is provided in Table 2, sequences of all plasmids are available upon request. Sequences of oligonucleotides employed for cloning, including primers for PCR to provide fragments for one-step gene replacements, are listed in Table S1. Double stranded DNA was custom-synthesized by ThermoFischer Scientific (Bremen, Germany).
Rich media were based on yeast extract (1%), peptone (2%) and the carbon source indicated. Glucose in rich medium was added at a concentration of either 2% or 4% glucose (YEPD). Ethanol was used at a concentration of 2%. For osmotic stabilization 1 M sorbitol was added as indicated. Synthetic media contained 0.67% yeast nitrogen base supplemented with ammonium sulfate, amino acids and bases as required, according to (Rose *et al.*, 1990) using 2% glucose or 2% ethanol as carbon sources.

Construction of deletion mutants and strains with tagged genes

Deletion mutants in *K. lactis* were obtained by one-step gene replacements as described for *S. cerevisiae* (Rothstein, 1991) either by directly using PCR products obtained with primers generating approximately 40 bp of homology flanking the genomic target sequences, or by a prior *in vivo* recombination with a plasmid-borne gene in *S. cerevisiae* to obtain longer flanking regions with homology to the target sequence. Genetic markers employed and procedures used have been essentially described in Heinisch *et al.* (2010) and complete

sequences of the chromosomal loci obtained after deletion or tagging of genes can be provided upon request. In short, the *Klsnf1* deletion was obtained by PCR amplification of the *kanMX* marker cassette from pUG6 (Gueldener *et al.*, 2002) primed by the oligonucleotides 07.302/07.303. Heterozygous diploids in strain KHO70 improved for homologous recombination by carrying the homozygous *ku80::loxP* deletion (Heinisch *et al.*, 2010) were selected on medium containing 100 mg/l G418, verified by PCR and subjected to tetrad analysis. One haploid segregant carrying the *Klsnf1::kanMX* allele, named KHO77-3C, was chosen for further crossings to eliminate the *ku80::loxP* marker.

For deletion of *KlMPK1* a similar approach was used, amplifying the *ScLEU2* marker cassette from pJJH955L with the oligonucleotide pair 13.253/13.254. The *Klbck1* deletion was generated with the *kanMX* marker cassette from pUG6 amplified with the oligonucleotides 11.422/11.423 as primers. One wild-type allele of *KlRLM1* in the haploid strain KHO69-8C was replaced by the *ScURA3* marker cassette amplified from pJJH955U with the oligonucleotide pair 05.103/05.104. For the deletion of *KlPFK1* and *KlPFK2*, the *ScLEU2* marker was used, amplified with the oligonucleotides 12.090/12.091 (*KlPFK1*) and 12.092/12.093 (*KlPFK2*), respectively. To generate the *Klmig1* and *Klsnf4* deletions, a *kanMX* cassette was amplified from pUG6 with the primer pairs 07.324/07.325 (*KlMIG1*) and 07.304/07.305 (*KlSNF4*), respectively. For the deletion of *KlPGI1*, the *ScURA3* marker cassette from pJJH955U was amplified with the oligonucleotides 13.320/13.321. The *Klgal83* deletion was generated with the *ScHIS3* marker cassette from pJJH955H amplified with the oligonucleotide pair 07.306/07.307.

The genomic *KlMIG1-3xHA* fusion was constructed by *in vivo* recombination employing a PCR product obtained with the oligonucleotides 14.271/14.272 from template pYM14 (Janke *et al.*, 2004).

The sequence encoding a TAP-tag was attached to the 3'-end of the *KlMIG1* open reading frame by amplification of the CM-BP-IgG-ProtA coding sequence from pBS1479 (Puig *et al.*, 2001) with the oligonucleotide pair 15.353/15.354. pRRO.347 was linearized 3' to the *KlMIG1* insert by restriction with SalI and introduced together with the PCR product into *S. cerevisiae* strain DHD5 for homologous recombination to yield pJJH1969. Transformants were selected on uracil-free medium and plasmids isolated from them were verified by restriction and sequence analyses.

For deletion of *ScMIG1* the *SpHIS5* marker cassette from pUG27 (Gueldener *et al.*, 2002) was amplified with the oligonucleotides 10.290/10.291, and used for a one-step gene replacement in BY4743 (Brachmann *et al.*, 1998), selecting on histidine-free medium. The genomic *ScMIG1-3xHA* fusion was obtained by amplification of the *3HA-kanMX* cassette

from pFA6a-3HA-kan (Longtine *et al.*, 1998) with the oligonucleotides 14.038/14.039 and introduced in the diploid strain BY4743, selecting for G418 resistance.

Serial drop dilution assays

To examine phenotypes of yeast strains in serial drop dilution assays, cells were grown overnight in rich medium, containing 1 M sorbitol if required. The cultures were diluted to an OD_{600} of 0.25 and grown for 3-5 h. Exponentially growing cells were adjusted to an OD_{600} of 0.1 with fresh growth medium and dilutions from 10^0 to 10^{-3} were prepared. 3 µl of each dilution were spotted onto plates containing stress agents as indicated and incubated for 2-3 days at 30℃, unless noted otherwise.

Fluorescence microscopy

For standard microscopic examination, cells were grown to early logarithmic phase in synthetic medium. The setup used for fluorescence microscopy consisted of a Zeiss Axioplan2e (Carl Zeiss, Jena, Germany), equipped with a 100x alpha-Plan Fluar objective (NA 1,45) and differential interference contrast (DIC). Images were acquired using an ORCA-Flash 4.0 LT Digital CMOS camera C11440 (Hamamatsu Photonics K. K., Japan). The setup was controlled by the Metamorph v6.2 program (Universal Imaging Corporation, Downingtown, USA). Brightfield images were acquired as single planes using differential358 interference-contrast (DIC). All fluorescence images were from single focal planes and scaled using Metamorphs scale image command. The processed images were overlayed using Metamorphs overlay images command.

Electron microscopy

For transmission electron microscopy (TEM) cells were prepared and imaged as described, previously (Backhaus *et al.*, 2013).

Metabolite determinations

Cells were grown overnight in 100 ml YEPD with 4% glucose to reach an OD_{600} of approximately 2. They were harvested by filtration using cellulose acetate filter with a pore size of 1.2 µm and washed once with ice-cold imidazole buffer (50 mM, pH 7.0).
The filter with the cells was then emerged and suspended in ethanol pre-heated to 80℃ and equilibrated with 20 mM imidazole buffer (pH 7.0) to quickly halt metabolism and extract metabolites. After 2 min incubation at 80℃ and dry ing to completeness in a rotary evaporator “Laborota 4000 efficient” (Heidolph Instruments GmbH, Schwabach, Germany),

metabolites were dissolved in 4 ml of ice-cold imidazole buffer (50 mM, pH 7.0) and debris was removed by centrifugation at 1700 g for 10 min. The glucose-6-phosphate concentration in the supernatant (= metabolite extract) was then determined enzymatically by coupling the reaction of glucose-6-phosphate dehydrogenase (from yeast, Roche) added at a final concentration of 3.5 U/ml to $NADP^+$ reduction (0.4 mM), following the increase in absorption at 340 nm. Fructose-6-phosphate was then determined by adding phosphoglucose isomerase (from yeast, Roche, at 7 U/ml) and determination of the additional increase in absorption. Fructose-1,6-bisphosphate concentration was determined in a similar fashion, by coupling its turnover to NADH oxidation with the auxiliary enzymes triosephosphate isomerase (from rabbit muscle, Sigma, 20 U/ml), and glycerol-3-phosphate dehydrogenase (from rabbit muscle, Roche, 3.5 U/ml), and after completion of the reaction of the triosephosphates addition of aldolase (from rabbit muscle, Roche, 0.5 U/ml) and determination of the absorption difference at 340 nm. For calculations, it was taken into account that one molecule of fructose-1,6-bisphosphate yields two molecules of NAD^+ in this case. Yeast dry weight was calculated assuming that 1 OD_{600} equals 0.36 mg/ml of cell dry weight, a value which was obtained from a standard curve plotting OD_{600} against the dry weight determined from 25-100 ml cultures of the *K. lactis* strains KDR1-9B, KDR22-8A and KDR24-7A at different growth stages.

Western blot analysis to determine the Mig1 phosphorylation state

Cells of either *K. lactis* (strains KDR65-1D and KDR67-3C) or *S. cerevisiae* (strains BDR30-3D and BDR32-1A) were grown overnight with shaking at 180 rpm at 30 ℃ in YEPD medium. 10^7 cells were sampled from logarithmically growing cultures as a control for non-phosphorylated Mig1, assuming that an OD_{600} of 1.0 equals $2x10^7$ cells/ml. To induce Mig1 phosphorylation, 10 ml of the culture were collected by centrifugation (1500 g for 3 min), resuspended in rich medium with 2% ethanol, incubated with shaking at 30 ℃ and harvested after 1 h of incubation. To the remaining overnight culture Caspofungin or Calcofluor white were added in different concentrations as indicated and incubated for 40 min at 30℃. 10^7 cells of each sample were then harvested by centrifugation (3700 g for 1 min, 4℃) and all samples were frozen in liquid nitrogen. The frozen cell pellets were resuspended in 500 µl “Roedel-mixture” [0.25 N NaOH, ß-mercaptoethanol, 100 mM phenylmethylsulfonyl fluoride (PMSF), 25x Complete Protease Inhibitor (Roche, Mannheim), 100 mM sodium orthovanadate, 10x phosphatase inhibitor mix (Roche, Mannheim)] and incubated on ice for 10 min. After addition of trichloroacetic acid to a final concentration of 13%, the samples were incubated on ice for another 10 min. The insoluble material was collected by centrifugation (20000 g for 10 min, 4℃), washed wi th 1 ml of cold acetone and dried at 55℃.

After resuspension in SDS sample buffer (60 mM Tris/HCl, 10% Glycerin, 2% SDS, 0,005% Bromphenol Blue), the samples were heated to 98°C for 3 min and loaded onto a 10% SDS-polyacrylamide gel. After completion of electrophoresis, gels were transferred to a nitrocellulose membrane (Whatman GmbH, Dassel) with the "Trans-Blot SD Semi-Dry Electrophoretic Transfer Cell" (Bio-Rad). Membranes were blocked for 1 to 3 h at room temperature with TBS-T (20 mM Tris, 150 mM NaCl, pH adjusted to 7.6 with HCl, 0.05% Tween 20) containing 3% bovine serum albumin (BSA, Roth, Karlsruhe, Germany). Mig1 was detected with anti-HA mouse antibody (kindly provided by Anja Lorberg, Osnabrück), which was diluted 1:10000 in TBS-T with 3% BSA. The membrane was incubated with the first antibody over night at 4°C. This step was followed by incubation with an anti-goat antibody from mouse coupled to IRDye 800CW (Li-Cor Biosciences, Lincoln, NE, USA; diluted 1:5000 in TBS-T with 3% BSA) for 1 h at room temperature. As an internal loading control, the phosphofructokinase subunits were detected using an anti-PFK polyclonal antiserum from rabbit at a dilution of 1:5000 (Heinisch, 1986, shown to also detect the PFK subunits from *K. lactis* in Heinisch *et al.*, 1993), which was applied for 4 h at room temperature, followed by the incubation with an anti-rabbit antibody from donkey coupled to IRDye 700DX (Rockland Immunochemicals, Gilbertsville, PA, USA, diluted 1:5000 with 3% BSA in TBS-T) for 1 h at room temperature. Labelled proteins were visualized and quantified with the "Odyssey Infrared Imaging System" (Li-Cor, Lincoln, NE, USA).

Tandem Affinity Purification (TAP)

Cells were grown overnight in synthetic medium without uracil. 10^7 cells of logarithmically growing cultures were collected by centrifugation, resuspended in rich medium with 2% ethanol and harvested after another 7 h of incubation with shaking at 30 °C. Cells were washed once with 50 ml cold water and then once with 25 ml cold 1x lysis buffer (LB; 0.3 M NaCl, 50 mM Hepes, 1.5 mM $MgCl_2$, 0.15% IGEPAL C-630) containing 0.5 mM PMSF. Cells were collected in a 50 ml Falcon tube and pelleted for 10 min at 3000 g. Supernatant was discarded and the pellets were dropped into liquid nitrogen. Frozen cell drops were stored at -80°C until further use. For lysis, drops were grinded in a Retsch MM301 mill for 30 s and the resulting powder was resuspended in 10 ml 1xLB containing 0.5 mM PMSF, 1 mM dithiothreotiol (DTT) and protease inhibitor mix FY (Serva Electrophoresis GmbH, Heidelberg, Germany). Lysates were then centrifuged at 3000 g for 10 min to remove cell debris and the supernatant was subjected to ultracentrifugation for 1 h at 100000 g. After that the greasy top phase was removed and the clear lysate was transferred to a new Falcon tube. IgG beads from a slurry with a calculated 0.2 ml/sample were washed three times with 10 ml cold 1x LB and added to the lysates, followed by an incubation for 1 h at 4°C with

constant mixing. Beads were then collected at 600-800 g for 3 min and the supernatant was removed. Beads were transferred to a Mobicol column (MoBiTec, Göttingen, Germany) and washed with 15 ml 1x LB containing 0.5 mM DTT. For protein elution, 150 µl 1x LB containing 0.5 mM DTT and 4 µl of TEV protease (1 mg/ml) were added and samples were incubated for 1 h at 16℃. After removing the beads by centrifugation the Mobicol column was washed with another 50 µl of 1x LB containing 0.5 mM DTT. The resulting eluate was precipitated by adding TCA to a final concentration of 13% and samples were incubated for 10 min on ice and again pelleted. Pellets were washed twice with 1 ml of cold acetone and dried at 55℃. After resuspension in SDS sample buffer, the samples were incubated at 95℃ for 3 min and separated in a 10% SDS-polyacrylamide gel. After the gel run the gel was stained with Coomassie to detect the protein bands.

Mass spectrometry

For mass spectrometry of KlMig1 the Coomassie-stained protein band was cut out of the gel. Gel slices were transferred into Eppendorf cups and washed twice with 250 µl ultra pure water and shaken for 10 min at 25℃ in each step. 250 µl of destaining solution (30% acetonitrile in 100 mM NH_4HCO_3) were added and slices were incubated for another 10 min at 25℃. This step was repeated until the gel slices were colorless. They were washed again with 250 µl ultra pure water followed by the addition of 250 µl acetonitrile and gently shaking for 15 min at 25℃. 100 µl reducing solution (10 mM DTT in 100 mM NH_4HCO_3) were added to the dried gel slices and incubated at 25℃ for 5 min, followed by a further incubation for 30 min at 50℃. After drying the gel piece again with acetonitrile, 100 µl alkylating solution (54 mM iodoacetamide in 100 mM NH_4HCO_3) were added and samples were incubated for 15 min in the dark. Gel slices were again incubated with the destaining solution and afterwards with acetonitrile. When acetonitrile was completely removed, 50-100 µl of freshly prepared Trypsin digest solution (50 mM NH_4HCO_3 with 5% acetonitrile and 0.01 mg/ml trypsin) was added and incubated overnight at 37℃, after which the supernatant was removed and stored at room temperature. Another 30 µl of acetonitrile were added to the gel slices and shaken for 20-30 min. The tube was then placed for 3 min in an ultrasonic bath and the supernatant was transferred into a new cup. This acetonitrile and the supernatant from the overnight incubation with trypsin were evaporated by a vacuum concentrator. Dried peptides were dissolved and applied to the mass spectrometry. Samples were loaded onto a trap column (Acclaim PepMap C18, 5 µm, 0.1 × 20 mm, Thermo Scientific, Sunnyvale, CA, USA) and washed. The trap column was switched in line with a separation column (Acclaim PepMap C18 2 µm, 0.075 × 150 mm, Thermo Scientific). Subsequently, bound substances were eluted by changing the mixture of buffer A (99% water, 1% acetonitrile, 0.1% formic acid) and

buffer B (80% acetonitrile, 20% water and 0.1% formic acid) from 100:0 to 20:80 within 45 min. The flow rate was kept constant at 0.3 µl/min. Successively eluted compounds were analyzed with ESI-ion trap (Amazon ETD Speed) with a captive spray ionization unit (Bruker Corporation, Billerica, MA, USA) by measuring the masses of the intact molecules as well as the masses of the fragments, which were generated of the corresponding parent ion by alternating collision-induced dissociation (CID) and electron transfer dissociation (ETD).

Acknowledgements: We are grateful to Bernadette Sander-Turgut and Andrea Murra for excellent technical support in Western analyses, protein purifications and metabolite determinations. This work was funded by a grant from the Deutsche Forschungsgemeinschaft (He 1880/6-1).

Table 1: Yeast strains used in this work.

A) *K. lactis*

Strain	Genotype	Reference
KDR1-9B	*MATa ura3 leu2 his3::loxP*	Rippert *et al.*, 2014
KDR1-1C	*MATα ura3 leu2 his3::loxP snf1::kanMX*	This work
KDR1-1D	*MATa ura3 leu2 his3::loxP lac4::loxP snf1::kanMX*	This work
KDR1-2A	*MATα ura3 leu2 his3::loxP lac4::loxP*	This work
KDR6-3A	*MATa ura3 leu2 his3::loxP bck1::kanMX*	This work
KDR8-9C	*MATa ura3 leu2 his3::loxP bck1::kanMX snf1::kanMX*	This work
KDR18-8A	*MATa ura3 leu2 his3::loxP rlm1::ScURA3*	This work
KDR18-10B	*MATα ura3 leu2 his3::loxP rlm1::ScURA3 snf1::kanMX*	This work
KDR19-2D	*MATa ura3 leu2 his3::loxP snf1::kanMX*	This work
KDR20-1A	*MATa ura3 leu2 his3::loxP*	This work
KDR20-6B	*MATa ura3 leu2 his3::loxP snf1::kanMX*	This work
KDR21-2D	*MATα ura3 leu2 his3::loxP snf1::kanMX*	This work
KDR22-8A	*MATα ura3 leu2 his3::loxP pfk1::ScLEU2*	This work
KDR22-1C	*MATα ura3 leu2 his3::loxP pfk1::ScLEU2 snf1::kanMX*	This work
KDR24-7A	*MATa ura3 leu2 his3::loxP pfk2::ScLEU2*	This work
KDR24-1C	*MATα ura3 leu3 his3::loxP pfk2::ScLEU2 snf1::kanMX*	This work
KDR30-1D	*MATα ura3 leu2 his3::loxP pfk1::ScLEU2 pfk2::ScLEU2*	This work
KDR50-4C	*MATα ura3 leu2 his3::loxP pgi1::ScURA3*	This work
KDR57-1D	*MATα ura3 leu2 his3::loxP pgi1::ScURA3 snf1::kanMX*	This work
KDR57-6A	*MATa ura3 leu2 his3::loxP pgi1::ScURA3 snf1::kanMX*	This work
KDR61-1D	*MATa ura3 leu2 his3::loxP mig1::kanMX snf1::kanMX*	This work
KDR65-1D	*MATa ura3 leu2 KlMIG1-3HA-kanMX*	This work
KDR67-3C	*MATα ura3 leu2 his3::loxP snf1::kanMX KlMIG1-3HA-kanMX*	This work
KHO70	*MATa/MATα ura3/ura3 leu2/leu2 ADE2/ade2::loxP HIS3/his3::loxP ku80::loxP/ku80::loxP*	Heinisch *et al.*, 2010
KHO77-3C	*MATa ura3 leu2 his3::loxP snf1::kanMX ku80::loxP*	This work
KHO88-3D	*MATa ura3 leu2 snf4::kanMX*	This work
KHO97-8B	*MATa ura3 leu2 his3::loxP mig1::kanMX*	This work
KHO121-1C	*MATα ura3 leu2 his3::loxP gal83::HIS3*	This work
KHO203-4A	*MATα ura3 leu2 his3::loxP lac4::loxP mpk1::ScLEU2*	This work
KHO203-5C	*MATa ura3 leu2 his3::loxP mpk1::ScLEU2 snf1::kanMX*	This work

All strains are derived from the congenic series described in Heinisch *et al.* (2010), with most deletions primarily obtained in KHO70.

B) *S. cerevisiae*

Strain	Genotype	Reference
BY4743	*MATa/MATα his3Δ1/ his3Δ1 leu2Δ0/ leu2Δ0 met15Δ0/MET15 lys2Δ0/LYS2 ura3Δ0/ ura3Δ0*	Brachmann *et al.*, 1998
BY4741	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*	Brachmann *et al.*, 1998
BKB12	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 snf1::KlLEU2*	Backhaus *et al.*, 2013
BKB40-3A	*MATα his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 mig1::SpHIS5*	This work
BDR29-5A	*MATα his3Δ1 leu2Δ0 lys2Δ0 mig1::SpHIS5 snf1::KlLEU2*	This work
BDR30-3D	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 MIG1-3HA-kanMX*	This work
BDR32-1A	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 snf1::KlLEU2 MIG1-3HA-kanMX*	This work

Table 2: Plasmids constructed and employed in this work.

Plasmid	Replication origins and selection markers	Gene, fragment	Cloning strategies
pDR61	*Klori, KlCEN2, URA3, Amp^R*	*ScSNF1*	EcoRI/BamHI fragment of pKBO.44[1] cloned into pCXJ18[2]
pDR64	*Klori, ScCEN4/ARS1, URA3, Amp^R*	*KlMIG1-3HA*	*3xHA-kanMX* amplified with primers 14.271/14.272 and introduced by *in vivo* recombination into pRRO.347
pDR65	*Kan^R*	*KlMIG1-3HA*	SalI/HpaI fragment of pDR64 cloned into pUK1921[3]
pDR81	*Klori, ScCEN4/ARS1, URA3, Amp^R*	*KlMIG1sa6-GFP-kanMX*	*GFP-kanMX* amplified with primers 07.247/15.182 from pRRO.366 and introduced by *in vivo* recombination in pJJH1879.3
pRRO.167	*Klori, ScCEN4/ARS1, URA3, Amp^R*	*KlSNF1*	*KlSNF1* amplified with primers 07.250/07.251 and cloned as SalI/HindIII into pCXJ22[2]
pRRO.345	*Kan^R*	*KlMIG1*	*KlMIG1* amplified with primers 08.262/08.263 and cloned as EcoRI/SalI into pUK1921[3]
pRRO.346	*Klori, KlCEN2, URA3, Amp^R*	*KlMIG1*	EcoRI/SalI fragment of RRO.345 cloned into pCXJ18[2]
pRRO.347	*Klori, ScCEN4/ARS1, URA3, Amp^R*	*KlMIG1*	EcoRI/SalI fragment of pRRO.345 cloned into pCXJ22[2]
pRRO.366	*Klori, ScCEN4/ARS1, URA3, Amp^R*	*KlMIG1-GFP-kanMX*	*GFP-kanMX* amplified with primers 08.264/07.247 and introduced by *in vivo* recombination into pRRO.347
pJJH1150	*Klori, KlCEN2, LEU2, Amp^R*	*KlPGK1p-GAL4bd-mCherry*	obtained by multiple cloning and *in vivo* recombination steps in the backbone of pCXJ20[2] carrying the first 72 codons of the Gal4 binding domain obtained from pGBD-C1[4] and 770 bp of the *KlPGK1* promoter preceding the translation start codon
pJJH1151	*Klori, ScCEN4/ARS1, LEU2, Amp^R*	*KlPGK1p-GAL4bd-mCherry*	derivative of pCXJ24[2] carrying the same insertions as pJJH1150
pJJH1879.1	*Klori, ScCEN4/ARS1, URA3, Amp^R*	*KlMIG1sa7*	pRRO.347 digested with BlpI and used for *in vivo* recombination with a synthesized *KlMIG1* fragment with exchange of a total of seven serine residues at three possible SNF1 target sites for alanines
pJJH1879.3	*Klori, ScCEN4/ARS1, URA3, Amp^R*	*KlMIG1sa6*	same as pJJH1879.1 but with the first serine residue (Ser245) not being exchanged for alanine in the *in vivo* recombination product
pJJH1995.10	*Klori, ScCEN4/ARS1, URA3, Amp^R*	*KlMIG1sa7-GFP-kanMX*	pDR81 digested with NheI/BlpI and the coding fragment substituted for the respective fragment from pJJH1879.1
pJJH1969	*Klori, ScCEN4/ARS1, URA3, Amp^R*	*KlMIG1-TAP*	TAP amplified with primers 15.353/15.354 from pBS1479[5] and introduced by *in vivo* recombination into pRRO.347

For cloning of the wild-type genes, genomic DNA preparations from *S. cerevisiae* or *K. lactis* were used as templates for PCR amplifications with the indicated primer pairs (see Table S1 for sequences). Vectors and plasmids employed for subcloning have been described in: [1]Backhaus *et al.*, 2013; [2]Chen, 1996; [3]Heinisch *et al.*, 1993; [4]James *et al.*, 1996; [5]Puig *et al.*, 2001

References

Ahuatzi, D., Riera, A., Pelaez, R., Herrero, P. and Moreno, F. (2007). Hxk2 regulates the phosphorylation state of Mig1 and therefore its nucleocytoplasmic distribution. *J. Biol. Chem.* **282**, 4485–4493.

Ashrafi, K., Lin, S. S., Manchester, J. K. and Gordon, J. I. (2000). Sip2p and its partner Snf1p kinase affect aging in *S. cerevisiae*. *Genes Dev.* **14**, 1872–85.

Backhaus, K., Heilmann, C. J., Sorgo, A. G., Purschke, G., de Koster, C. G., Klis, F. M. and Heinisch, J. J. (2010). A systematic study of the cell wall composition of *Kluyveromyces lactis*. *Yeast* **27**, 647–660.

Backhaus, K., Buchwald, U., Heppeler, N., Schmitz, H.-P., Rodicio, R. and Heinisch, J. J. (2011). Milk and sugar: Regulation of cell wall synthesis in the milk yeast *Kluyveromyces lactis*. *Eur. J. Cell Biol.* **90**, 745–750.

Backhaus, K., Rippert, D., Heilmann, C. J., Sorgo, A. G., de Koster, C. G., Klis, F. M., Rodicio, R. and Heinisch, J. J. (2013). Mutations in SNF1 complex genes affect yeast cell wall strength. *Eur. J. Cell Biol.* **92**, 383–395.

Brachmann, C. B., Davies, A., Cost, G. J., Caputo, E., Li, J., Hieter, P. and Boeke, J. D. (1998). Designer deletion strains derived from *Saccharomyces cerevisiae* S288C: A useful set of strains and plasmids for PCR-mediated gene disruption and other applications. *Yeast* **14**, 115–132.

Carlson, M., Osmond, B. C. and Botstein, D. (1981). Mutants of yeast defective in sucrose utilization. *Genetics* **98**, 25–40.

Cassart, J. P., Georis, I., Ostling, J., Ronne, H. and Vandenhaute, J. (1995). The *MIG1* repressor from *Kluyveromyces lactis*: cloning, sequencing and functional analysis in *Saccharomyces cerevisiae*. *FEBS Lett.* **371**, 191–194.

Chen, X. J. (1996). Low- and high-copy-number shuttle vectors for replication in the budding yeast *Kluyveromyces lactis*. *Gene* **172**, 131–136.

Ciriacy, M. and Breitenbach, I. (1979). Physiological effects of seven different blocks in glycolysis in *Saccharomyces cerevisiae*. *J. Bacteriol.* **139**, 152–60.

DeVit, M. J. and Johnston, M. (1999). The nuclear exportin Msn5 is required for nuclear export of the Mig1 glucose repressor of *Saccharomyces cerevisiae*. *Curr. Biol.* **9**, 1231–41.

DeVit, M. J., Waddle, J. A. and Johnston, M. (1997). Regulated nuclear translocation of the Mig1 glucose repressor. *Mol. Biol. Cell* **8**, 1603–1618.

Dong, J. and Dickson, R. C. (1997). Glucose represses the lactos-galactose regulon in *Kluyveromyces lactis* through a SNF-1 and MIG1-dependent pathway that modulates galactokinase (GAL1) gene expression. *Nucleic Acids Res.* **25**, 3657–3664.

Gancedo, J. M. (1998). Yeast carbon catabolite repression. *Microbiol. Mol. Biol. Rev.* **62**, 334–61.

Georis, I., Cassart, J. P., Breunig, K. D. and Vandenhaute, J. (1999). Glucose repression of the *Kluyveromyces lactis* invertase gene *KlINV1* does not require Mig1p. *Mol. Gen. Genet.* **261**, 862–70.

Goffrini, P., Wésolowski-Louvel, M. and Ferrero, I. (1991). A phosphoglucose isomerase gene is involved in the Rag phenotype of the yeast *Kluyveromyces lactis*. *MGG Mol. Gen. Genet.* **228**,.

Goffrini, P., Ficarelli, A., Donnini, C., Lodi, T., Puglisi, P. P. and Ferrero, I. (1996). *FOG1* and *FOG2* genes, required for the transcriptional activation of glucose-repressible genes of *Kluyveromyces lactis*, are homologous to *GAL83* and *SNF1* of *Saccharomyces cerevisiae*. *Curr. Genet.* **29**, 316–26.

Gueldener, U., Heinisch, J., Koehler, G. J., Voss, D. and Hegemann, J. H. (2002). A second set of loxP marker cassettes for Cre-mediated multiple gene knockouts in budding yeast. *Nucleic Acids Res.* **30**, e23.

Hardie, D. G. (2003). Minireview: The AMP-activated protein kinase cascade: The key sensor of cellular energy status. *Endocrinology* **144**, 5179–5183.

Hedbacker, K. and Carlson, M. (2006). Regulation of the nucleocytoplasmic distribution of Snf1-Gal83 protein kinase. *Eukaryot. Cell* **5**, 1950–6.

Hedbacker, K. and Carlson, M. (2008). SNF1/AMPK pathways in yeast. *Front. Biosci.* **13**, 2408–20.

Heinisch, J. J. (1986). Construction and physiological characterization of mutants disrupted in the phosphofructokinase genes of *Saccharomyces cerevisiae*. *Curr. Genet.* **11**, 227–234.

Heinisch, J. J. (2005). Baker's yeast as a tool for the development of antifungal kinase inhibitors - targeting protein kinase C and the cell integrity pathway. *Biochim. Biophys. Acta - Proteins Proteomics* **1754**, 171–182.

Heinisch, J. J. and Zimmerman, F. K. (1985). Is the phosphofructokinase-reaction obligatory for glucose fermentation by *Saccharomyces cerevisiae*? *Yeast* **1**, 173–175.

Heinisch, J. J., Kirchrath, L., Liesen, T., Vogelsang, K. and Hollenberg, C. P. (1993). Molecular genetics of phosphofructokinase in the yeast *Kluyveromyces lactis*. *Mol. Microbiol.* **8**, 559–570.

Heinisch, J. J., Buchwald, U., Gottschlich, A., Heppeler, N. and Rodicio, R. (2010). A tool kit for molecular genetics of *Kluyveromyces lactis* comprising a congenic strain series and a set of versatile vectors. *FEMS Yeast Res.* **10**, 333–42.

Hong, S. P. and Carlson, M. (2007). Regulation of Snf1 protein kinase in response to environmental stress. *J. Biol. Chem.* **282**, 16838–16845.

Honigberg, S. M. and Lee, R. H. (1998). Snf1 kinase connects nutritional pathways controlling meiosis in *Saccharomyces cerevisiae*. *Mol. Cell. Biol.* **18**, 4548–4555.

Jacoby, J., Hollenberg, C. P. and Heinisch, J. J. (1993). Transaldolase mutants in the yeast *Kluyveromyces lactis* provide evidence that glucose can be metabolized through the pentose phosphate pathway. *Mol. Microbiol.* **10**, 867–876.

Jacoby, J. J., Kirchrath, L., Gengenbacher, U. and Heinisch, J. J. (1999). Characterization of *KlBCK1*, encoding a MAP kinase kinase kinase of *Kluyveromyces lactis*. *J. Mol. Biol.* **288**, 337–52.

James, P., Halladay, J. and Craig, E. A. (1996). Genomic libraries and a host strain designed for highly efficient two-hybrid selection in yeast. *Genetics* **144**, 1425–1436.

Janke, C., Magiera, M. M., Rathfelder, N., Taxis, C., Reber, S., Maekawa, H., Moreno-Borchart, A., Doenges, G., Schwob, E., Schiebel, E., *et al.* (2004). A versatile toolbox for PCR-based tagging of yeast genes: new fluorescent proteins, more markers and promoter substitution cassettes. *Yeast* **21**, 947–962.

Jendretzki, A., Wittland, J., Wilk, S., Straede, A. and Heinisch, J. J. (2011). How do I begin? Sensing extracellular stress to maintain yeast cell wall integrity. *Eur. J. Cell Biol.* **90**, 740–744.

Jung, U. S. and Levin, D. E. (1999). Genome-wide analysis of gene expression regulated by the yeast cell wall integrity signalling pathway. *Mol. Microbiol.* **34**, 1049–1057.

Kirchrath, L., Lorberg, A., Schmitz, H.-P., Gengenbacher, U. and Heinisch, J. J. (2000). Comparative genetic and physiological studies of the MAP kinase Mpk1p from *Kluyveromyces lactis* and *Saccharomyces cerevisiae*. *J. Mol. Biol.* **300**, 743–58.

Klis, F. M., Mol, P., Hellingwerf, K. and Brul, S. (2002). Dynamics of cell wall structure in *Saccharomyces cerevisiae*. *FEMS Microbiol. Rev.* **26**, 239–256.

Kock, C., Dufrêne, Y. F. and Heinisch, J. J. (2015). Up against the wall: Is yeast cell wall integrity ensured by mechanosensing in plasma membrane microdomains? *Appl. Environ. Microbiol.* **81**, 806–811.

Lamas-Maceiras, M., Rodríguez-Belmonte, E., Becerra, M., González-Siso, M. I. and Cerdán, M. E. (2015). KlGcr1 controls glucose-6-phosphate dehydrogenase activity and responses to H_2O_2, cadmium and arsenate in *Kluyveromyces lactis*. *Fungal Genet. Biol.* **82**, 95–103.

Levin, D. E. (2005). Cell wall integrity signaling in *Saccharomyces cerevisiae*. *Microbiol Mol Biol Rev* **69**, 262–291.

Lodi, T., Saliola, M., Donnini, C. and Goffrini, P. (2001). Three target genes for the transcriptional activator Cat8p of *Kluyveromyces lactis*: acetyl coenzyme A synthetase genes *KlACS1* and *KlACS2* and lactate permease gene *KlJEN1*. *J Bacteriol* **183**, 5257–5261.

Longtine, M. S., Mckenzie III, A., Demarini, D. J., Shah, N. G., Wach, A., Brachat, A., Philippsen, P. and Pringle, J. R. (1998). Additional modules for versatile and economical PCR-based gene deletion and modification in *Saccharomyces cerevisiae*. *Yeast* **14**, 953–961.

Mates, N., Kettner, K., Heidenreich, F., Pursche, T., Migotti, R., Kahlert, G., Kuhlisch, E., Breunig, K. D., Schellenberger, W., Dittmar, G., *et al.* (2014). Proteomic and functional consequences of hexokinase deficiency in glucose-repressible *Kluyveromyces lactis*. *Mol. Cell. Proteomics* **13**, 860–875.

Mehlgarten, C., Krijger, J.-J., Lemnian, I., Gohr, A., Kasper, L., Diesing, A.-K., Grosse, I. and Breunig, K. D. (2015). Divergent evolution of the transcriptional network controlled by Snf1-interacting protein Sip4 in budding yeasts. *PLoS One* **10**, e0139464.

Merico, A., Galafassi, S., Piskur, J. and Compagno, C. (2009). The oxygen level determines the fermentation pattern in *Kluyveromyces lactis*. *FEMS Yeast Res.* **9**, 749–756.

Nehlin, J. O., Carlberg, M. and Ronne, H. (1991). Control of yeast *GAL* genes by *MIG1* repressor: a transcriptional cascade in the glucose response. *EMBO J.* **10**, 3373–7.

Östling, J. and Ronne, H. (1998). Negative control of the Mig1p repressor by Snf1p-dependent phosphorylation in the absence of glucose. *Eur. J. Biochem.* **252**, 162–168.

Philip, B. and Levin, D. E. (2001). Wsc1 and Mid2 are cell surface sensors for cell wall integrity signaling that act through Rom2, a guanine nucleotide exchange factor for Rho1. *Mol. Cell. Biol.* **21**, 271–280.

Portillo, F., Mulet, J. M. and Serrano, R. (2005). A role for the non-phosphorylated form of yeast Snf1: tolerance to toxic cations and activation of potassium transport. *FEBS Lett.* **579**, 512–516.

Prior, C., Mamessier, P., Fukuhara, H., Chen, X. J. and Wesolowski-Louvel, M. (1993). The hexokinase gene is required for transcriptional regulation of the glucose transporter gene *RAG1* in *Kluyveromyces lactis*. *Mol. Cell. Biol.* **13**, 3882–3889.

Puig, O., Caspary, F., Rigaut, G., Rutz, B., Bouveret, E., Bragado-Nilsson, E., Wilm, M. and Séraphin, B. (2001). The tandem affinity purification (TAP) method: A general procedure of protein complex purification. *Methods* **24**, 218–229.

Rippert, D., Heppeler, N., Albermann, S., Schmitz, H.-P. and Heinisch, J. J. (2014). Regulation of cytokinesis in the milk yeast *Kluyveromyces lactis*. *Biochim. Biophys. Acta* **1843**, 2685–97.

Rodicio, R. and Heinisch, J. J. (2010). Together we are strong - cell wall integrity sensors in yeasts. *Yeast* **27**, 531–540.

Rodicio, R. and Heinisch, J. J. (2013). Yeast on the milky way: genetics, physiology and biotechnology of *Kluyveromyces lactis*. *Yeast* **30**, 165–177.

Rodicio, R., Buchwald, U., Schmitz, H.-P. and Heinisch, J. J. (2008). Dissecting sensor functions in cell wall integrity signaling in *Kluyveromyces lactis*. *Fungal Genet. Biol.* **45**, 422–35.

Rose, M., Winston, F., and Hieter, P. (1990). Methods in yeast genetics - A laboratory course manual. *Cold Spring Harbor Laboratory Press*, Cold Spring Harbor, New York. *ISBN 0-87969-354-1*.

Rothstein, R. (1991). Targeting, disruption, replacement, and allele rescue: integrative DNA transformation in yeast. *Methods Enzymol.* **194**, 281–301.

Saliola, M., Scappucci, G., De Maria, I., Lodi, T., Mancini, P. and Falcone, C. (2007). Deletion of the glucose-6-phosphate dehydrogenase gene *KlZWF1* affects both fermentative and respiratory metabolism in *Kluyveromyces lactis*. *Eukaryot. Cell* **6**, 19–27.

Schmitz, H.-P. and Heinisch, J. J. (2003). Evolution, biochemistry and genetics of protein kinase C in fungi. *Curr. Genet.* **43**, 245–254.

Schüller, H.-J. (2003). Transcriptional control of nonfermentative metabolism in the yeast *Saccharomyces cerevisiae*. *Curr. Genet.* **43**, 139–160.

Tarrío, N., Becerra, M., Cerdán, M. E. and González Siso, M. I. (2006). Reoxidation of cytosolic NADPH in *Kluyveromyces lactis*. *FEMS Yeast Res.* **6**, 371–380.

Treitel, M. A., Kuchin, S. and Carlson, M. (1998). Snf1 protein kinase regulates phosphorylation of the Mig1 repressor in *Saccharomyces cerevisiae*. *Mol. Cell. Biol.* **18**, 6273–80.

Vega, M., Riera, A., Fernández-Cid, A., Herrero, P. and Moreno, F. (2016). Hexokinase 2 is an intracellular glucose sensor of yeast cells that maintains the structure and activity of Mig1 protein repressor complex. *J. Biol. Chem.* **291**, 7267–7285.

Vincent, O., Townley, R., Kuchin, S. and Carlson, M. (2001). Subcellular localization of the Snf1 kinase is regulated by specific beta subunits and a novel glucose signaling mechanism. *Genes Dev.* **15**, 1104–14.

Ye, T., Elbing, K. and Hohmann, S. (2008). The pathway by which the yeast protein kinase Snf1p controls acquisition of sodium tolerance is different from that mediating glucose regulation. *Microbiology* **154**, 2814–26.

Ye, T., Bendrioua, L., Carmena, D., García-Salcedo, R., Dahl, P., Carling, D. and Hohmann, S. (2014). The mammalian AMP-activated protein kinase complex mediates glucose regulation of gene expression in the yeast *Saccharomyces cerevisiae*. *FEBS Lett.* **588**, 2070–2077.

Supplementary Material

Table S1: Oligonucleotides used.

Number	Sequence (5' → 3')	Gene
05.11	GGCGCTCGAGGTTTAGTGTTACAAATAAAAGCGG	KlRLM1
05.103	CCCATCGCCACAAGTTTTCATAAGACCTCTTTCTTTTAGCATACGATGGGCTTCGTAC GCTGCAGGTCGAC	KlRLM1
05.104	GGAATAACTCCTAATCTTCCTGTCATTTATCTACAGATCCTTTATCTGACCCGCATAG GCCACTAGTGGATCTG	KlRLM1
06.88	GCAATTTGTTAATTTGGGTATTGTTGG	KlBCK1
06.89	GGCGAGATCTGAATGGGTACAATACTTGCGTTTGG	KlBCK1
07.247	GGCAGTGAGCGCAACGCAATTAATGTGAGTTAGCTCACTCATTAGGCACCCCAGGC TTGAATTCGAGCTCGTTTAAAC	KlMIG1-GFP
07.248	GGCGGAATTCGTCAGACAGACGAAGCTGGAG	KlSNF4
07.249	GGCAGTCGACCAGGAAATGTGAGCAAATCCAC	KlSNF4
07.250	GGCGAAGCTTCGAGTGTTGTGAGCTAAGAGTA	KlSNF1
07.251	GGCAGTCGACACCTATCTGCATATCTGGATAG	KlSNF1
07.302	TCCCTCTGTTGAACGCAAACTCTTAGTGAAGCATTTACGAGTACATACTCATCAGAGA AGCTTCGTACGCTGCAGGTCGAC	KlSNF1
07.303	CCTTAAAATCAACTTCCTTGGCTATTCACTGCAAGTTCCATAATCAGCCTGGTTGCCC GCATAGGCCACTAGTGGATCTG	KlSNF1
07.304	ACAGATGCCTGCAAGCAGTGATAAACTTCAACCGAAAGATCAGCAGACCATTGAGCT CTTCGTACGCTGCAGGTCGAC	KlSNF4
07.305	AGGATATCGCTTAGAGTGAGAACACCAGTTAGGAATCCGTTCGAATCGACGACGCC GCATAGGCCACTAGTGGATCTG	KlSNF4
07.306	TGGCGGAGGTAGCATAGAGGAAAACGTTCAAAAGTACCAAGAGACTACCTGACTTC GTACGCTGCAGGTCGAC	KlGAL83
07.307	TCAAATCTCTAGGAGTGGTTTCATTGCAATGGCGCATATAGTATCTGTGTTGCCCGC ATAGGCCACTAGTGGATCTG	KlGAL83
07.324	GGTGACTGATACGAAAGGAATCTGGAACTTATAGCACCAATGACAGAGGCGACTTC GTACGCTGCAGGTCGAC	KlMIG1
07.325	AGTCATTATTTTGGTGGTAAAAGGTCTAAGCTTCGAAGTGATGGCAACTGCACTGGC CCGCATAGGCCACTAGTGGATCTG	KlMIG1
08.262	CGATATGCAATCTTCAGCTGAG	KlMIG1
08.263	CGCGGTCGACGTGTACCACGTGCGATTAGGTCAG	KlMIG1
08.264	CAGAATAAGCCAGTGCAGTTGCCATCACTTCGAAGCTTAGACCTTTTACCACCAAAA CGGATCCCCGGGTTAATTAA	KlMIG1-GFP
10.288	GGCCAAGAACGAGATGAAGG	ScMIG1
10.289	CATAGCTGCCACTAACCTAC	ScMIG1
10.290	CGAGAGTTGAGTATAGTGGAGACGACATACTACCATAGCCTTCGTACGCTGCAGGT CGAC	ScMIG1
10.291	TCTTTTGATTTATCTGCACCGCCAAAAACTTGTCAGCGTAGCATAGGCCACTAGTGG ATCTG	ScMIG1
11.040	TTCAATCCGCTAGATTTCCC	KlRLM1

11.422	TAGTATCTGATAAGTAAAGTCCCGCTAAATATAGATGAACCGTGGATGATCTTCGTACGCTGCAGGTCGAC	KlBCK1
11.423	ATTATTTGTTAGTGATTTAACGTTATATATCATTTAAAAAAGATCATGCTGCATAGGCCACTAGTGGATCTG	KlBCK1
12.090	AAAGCCTGTGTTTTAACGAAGAGAAAAGAACAAAATAAAAAGTAAACTTCGTACGCTGCAGGTCGAC	KlPFK1
12.091	TAATGTTCAGAACTTTTCATGACAGGTAAGAGGGTTAGTCTTGCATAGGCCACTAGTGGATCTG	KlPFK1
12.092	ATTACAAAAAGGAAAATATTGATACAAGTAATAATAAACTGCTTCGTACGCTGCAGGTCGAC	KlPFK2
12.093	ATTTCAGTGGGAATTTTGAGGAAGCAGATACGTTTTTAATTGCATAGGCCACTAGTGGATCTG	KlPFK2
12.094	GATTGATACGAACAAATGGAG	KlPFK1
12.095	gcgcgtcgaCAATAAAATAGTTGCATCACGTG	KlPFK1
12.096	GGCTACGAAAATGATCAAAAAGG	KlPFK2
12.097	TTTGGTGAATTCACTTGTTTACG	KlPFK2
13.253	ATATCAGTCCGGATCAACTGATTGTGCCCTTTTCTCCACCGAACTTCGTACGCTGCAGGTCGAC	KlMPK1
13.254	TACAAAGAGAGTGGATGAAGCTATATACCAGACGGTTTCATTAGCATAGGCCACTAGTGGATCTG	KlMPK1
13.320	GATAGATTCTAAAACAGAAATAAAAAGACAAATATCAGAACTTCGTACGCTGCAGGTCGAC	KlPGI1
13.321	CATATCTTAGGTGAAAAGGAAATTGCATCGCATTCTCACAGCATAGGCCACTAGTGGATCTG	KlPGI1
13.322	GCCAGATGCATGCGAACTTG	KlPGI1
13.323	gcgcggatccGCGTCCTCTATATGCTATGAG	KlPGI1
14.038	AAACATTACCACCCATAAGAAGTTTACCGTTGCCCTTCCCACACATGGACCGGATCCCCGGGTTAATTAA	ScMIG1-3HA
14.039	CTGGTAAAAATTATATTTTAGGGGGCCACAATAAAGTTAAAAACAACAGTGCATAGGCCACTAGTGGATCTG	ScMIG1-3HA
14.271	GTTGCCATCACTTCGAAGCTTAGACCTTTTACCACCAAAATCCGGTTCTGCTGCTAGATA	KlMIG1-3HA
14.272	GATATATAATTGATGGACAAGAGCTAGTAAAATTCTATATTGCATAGGCCACTAGTGGATCTG	KlMIG1-3HA
15.353	AGTTGCCATCACTTCGAAGCTTAGACCTTTTACCACCAAAAATGGAAAAGAGAAGATGGAAAAAG	TAP
15.354	ACACAGGAAACAGCTATGACCATGATTACGCCAAGCTTGGCCGCTCTAGAACTAGTGG	TAP

Underlined sequences designate restriction sites used for cloning or sequences with homology to cassettes from vectors used as PCR templates.

Table S2: Protein report of mass-spectrometry of KlMig1.

Range	Cmpd.	m/z meas.	Δ m/z [ppm]	Rt [min]	Scores	Site [%]	Sequence	Modifications	Type
111 - 134	208	780.3030	-81.75	27.47	56.9 (M:56.9)		R.NANDSAAVDSAGGTSANVIEPNHK.L		CID
111 - 134	236	1169.9080	-118.43	28.57	76.5 (M:76.5)		R.NANDSAAVDSAGGTSANVIEPNHK.L		ETD
138 - 154	258	618.2690	-73.46	29.56	80.3 (M:80.3)		K.STNSIKQDGSTFTEPLK.S		ETD
138 - 154	250	926.9100	-62.56	29.22	41.3 (M:41.3)		K.STNSIKQDGSTFTEPLK.S		CID
182 - 197	421	836.3920	-53.39	36.80	46.6 (M:46.6)		R.SQNNSGNIDLLLNAAK.F		ETD
182 - 197	421	836.3920	-53.39	36.80	109.3 (M:109.3)		R.SQNNSGNIDLLLNAAK.F		CID
247 - 257	233	391.8440	-151.04	28.45	30.0 (M:30.0)		R.AKLSALSSLQR.M		CID
247 - 257	233	391.8440	-151.04	28.45	71.7 (M:71.7)		R.AKLSALSSLQR.M		ETD
303 - 326	375	902.9900	-94.25	34.63	54.7 (M:54.7)	30.79	R.TRSWTNLSEQQSPSGFSSSALNSR.F	Phospho: 1	CID
305 - 326	394	1185.4500	-85.59	35.43	39.2 (M:39.2)		R.SWTNLSEQQSPSGFSSSALNSR.F		ETD
327 - 342	333	611.5810	-87.27	32.99	75.5 (M:75.5)		R.FSSSNSLNQLIDQHSR.N		ETD
327 - 342	333	611.5810	-87.27	32.99	44.4 (M:44.4)		R.FSSSNSLNQLIDQHSR.N		CID
343 - 354	395	625.3050	-77.63	35.52	55.9 (M:55.9)		R.NSSTVSISTLLK.Q		CID
343 - 354	395	625.3050	-77.63	35.52	44.1 (M:44.1)		R.NSSTVSISTLLK.Q		ETD
343 - 354	475	665.3430	9.46	39.05	32.4 (M:32.4)	44.02	R.NSSTVSISTLLK.Q	Phospho: 3	ETD
343 - 354	475	665.3430	9.46	39.05	55.3 (M:55.3)	54.27	R.NSSTVSISTLLK.Q	Phospho: 3	CID
343 - 354	602	705.2890	-43.77	44.05	28.0 (M:28.0)	54.01	R.NSSTVSISTLLK.Q	Phospho: 3, 4	CID
343 - 354	592	705.3050	-21.09	43.64	25.0 (M:25.0)	68.58	R.NSSTVSISTLLK.Q	Phospho: 3, 4	ETD
355 - 377	270	660.7570	-90.65	30.25	40.9 (M:40.9)		K.QETVISQDEDMSTEDAYGRPLKK.S		ETD
355 - 377	270	660.7570	-90.65	30.25	46.7 (M:46.7)		K.QETVISQDEDMSTEDAYGRPLKK.S		CID
467 - 474	314	441.7270	-93.69	32.18	48.2 (M:48.2)		R.SLDLLPPK.-		CID

Sequence and range of the detected peptides are given. The m/z meas. data indicate the mass of the mother-ion. Phosphorylation positions are shown in the modification section.

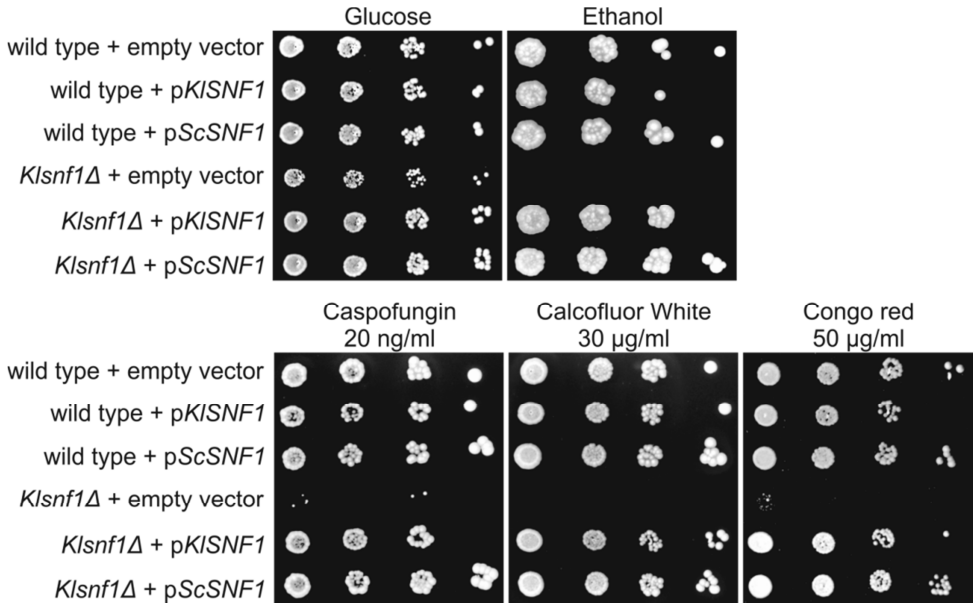


Figure S1: *ScSNF1* complements a *Klsnf1* deletion, inlcuding its cell wall defects. A wild-type strain of *K. lactis* (KDR1-9B) and a *Klsnf1* deletion (KDR1-1C) carrying the indicated plasmids were subjected to drop dilution assays and incubated at 30℃ on synthetic media con taining the cell wall stress agents as indicated. A plate with ethanol as sole carbon source was used as a control for KlSNF1 functions in central carbohydrate metabolism.

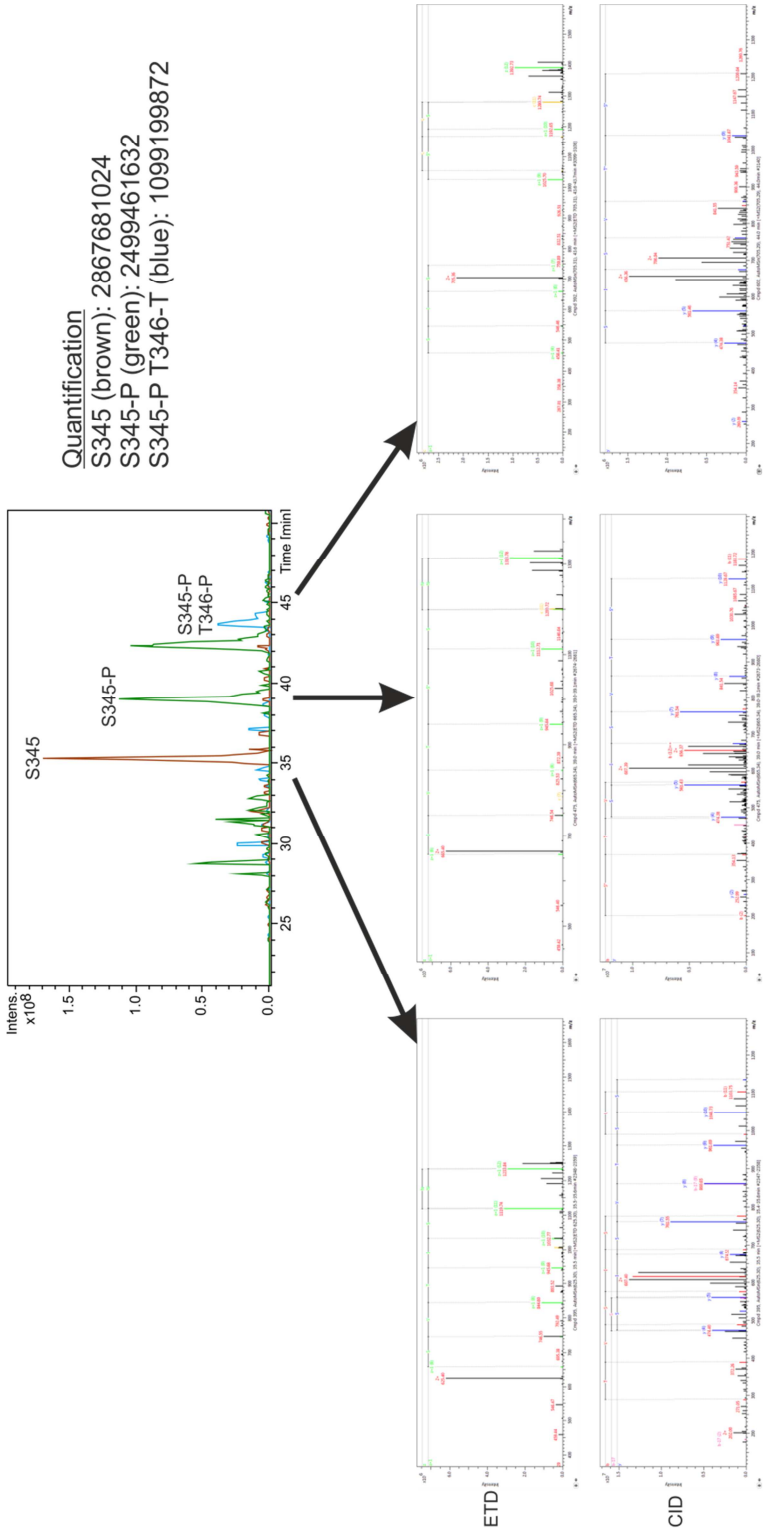


Figure S2: Mass-spectrometry data of the peptide NSSTVSISTLLK of KlMig1 (amino acids 343-354). The graphic above shows the HPLC data of one peptide, which was present in three different modification states. The brown peak desginates the unmodified peptide, which lacks phosphorylated residues. The green peak represents the peptide phosphorylated at Ser345, whereas the blue peak originates from dual phosphorylations at Ser345 and Thr346. The corresponding ETD and CID data are depicted in the panels below.

3.3 Regulation of cytokinesis in the milk yeast *Kluyveromyces lactis*

D. Rippert, N. Heppeler, S. Albermann, H.-P. Schmitz & J. J. Heinisch, (2014)

Biochimica et Biophysica Acta **1843:** 2685-2697

Abstract

Cytokinesis in yeast and mammalian cells is a highly coordinated process mediated by the constriction of an actomyosin ring. In yeasts, it is accompanied by the formation of a chitinous primary septum. Although much is known about the regulation of cytokinesis in budding yeast, overlapping functions of redundant genes complicates genetic analyses. Here, we investigated the effects of various deletion mutants on cytokinesis in the milk yeast *Kluyveromyces lactis*. To determine the spatiotemporal parameters of cytokinesis components, live-cell imaging of fluorophor-tagged KlMyo1 and a new Lifeact probe for KlAct1 was employed. In contrast to *Saccharomyces cerevisiae*, where deletion of *ScMYO1* is lethal, *Klmyo1* deletion was temperature-sensitive. Transmission and scanning electron microscopy demonstrated that the *Klmyo1* deletion cells had a defect in the formation of the primary septum and in cell separation; this result was confirmed by FACS analyses. Deletion of *KlCYK3* was lethal, whereas in *S. cerevisiae* a *cyk3* deletion is synthetically lethal with *hof1* deletion. Growth of *Klhof1* mutants was osmoremedial at 25℃, as it is in *S. cerevisiae*. *CYK3* and *HOF1* genes cross-complemented in both species, suggesting that they are functional homologs. Inn1, a common interactor for these two regulators, was essential in both yeasts and the encoding genes did not cross-complement. The C2 domain of the Inn1 homologs conferred species specificity. Thus, our work establishes *K. lactis* as a model yeast to study cytokinesis with less genetic redundancy than *S. cerevisiae*. The viability of *Klmyo1* deletions provides an advantage over budding yeast to study actomyosin-independent cytokinesis. Moreover, the lethality of *Klcyk3* null mutants suggests that there are fewer functional redundancies with KlHof1 in *K. lactis*.

DOI: 10.1016/j.bbamcr.2014.07.020

3.4 Investigation of the role of four mitotic septins and chitin synthase 2 for cytokinesis in *Kluyveromyces lactis*

D. Rippert & J. J. Heinisch, (2016)

Fungal Genetics and Biology **94:** 69-78

Abstract

Septins are key components of the cell division machinery from yeast to humans. The model yeast *Saccharomyces cerevisiae* has five mitotic septins, Cdc3, Cdc10, Cdc11, Cdc12, and Shs1. Here we characterized the five orthologs from the genetically less-redundant milk yeast *Kluyveromyces lactis*. We found that except for *KlSHS1* all septin genes are essential. *Klshs1* deletions displayed temperature-sensitive growth and morphological defects. Heterologous complementation analyses revealed that all five *K. lactis* genes encode functional orthologs of their *S. cerevisiae* counterparts. Fluorophore-tagged versions of the *K. lactis* septins localized to a ring at the incipient bud site and split into two separate rings at the bud neck later in cytokinesis. One of the key proteins recruited to the bud neck by septins in *S. cerevisiae* is the chitin synthase Chs2, which synthesizes the primary septum. *KlCHS2* was found to be essential and deletions showed cytokinetic defects upon spore germination. KlChs2-GFP also localized to the bud neck and to punctate structures in *K. lactis*. We conclude that cytokinesis in *K. lactis* is similar to *S. cerevisiae* and chimeric septin complexes are fully functional in both yeasts. In contrast to some *S. cerevisiae* strains, KlChs2 and KlCdc10 were found to be essential.

DOI: 10.1016/j.fgb.2016.07.007

4. Concluding remarks

Within the last years many studies were done on yeast cytokinesis and signal transduction related to high and low osmolarity and cell wall integrity (HOG and CWI pathway). Furthermore, the regulation of central carbohydrate metabolism has been extensively investigated. However, all these studies focus on the detailed regulation of each signaling pathway with very little attention being paid to the coordination of the different pathways which is necessary to ensure cell wall synthesis at the bud neck. This thesis aimed to find such common aspects among these complex regulations by using the budding yeast *S. cerevisiae* as an ideal model organism. For this organism entire deletion collections of all open reading frames and methods for its molecular handling are available. Due to the complexity of regulatory circuits and the disadvantage of high genetic redundancy in this yeast, parallel research was started in the genetically less redundant milk yeast *K. lactis*.

This thesis is structured around four manuscripts/publications, which are roughly divided into two subjects: two of the works report on the role of the SNF1 kinase complex in cell wall integrity and two others deal with the regulation of cytokinesis in *K. lactis*.

Cell wall synthesis is quite important for yeast cells, since the cell wall determines the shape of the cell, ensures the cell's integrity and serves as a first barrier against adverse environmental conditions. The cell wall consists of polysaccharides and proteins, whereby the former are synthesized from central sugar phosphate precursors. The first manuscript (Backhaus *et al.*, 2013) showed that the intracellular concentration of such sugar phosphates is influenced by the SNF1/AMPK complex, which was also found to act in parallel to CWI signaling in *S. cerevisiae*. It was speculated that a decrease of these sugar phosphates in the *snf1* deletion could explain the observed thinner cell wall, as well as the suppression of the cell integrity phenotypes of this deletion by an additional *pfk1* deletion, leading to an increase in glucose- and fructose-6-phosphate concentration. However, if and how downstream SNF1 effectors could also play a role remained an open question.

This problem was followed-up in the second manuscript (Rippert *et al.*, 2016), in which *K. lactis* was used as a model. SNF1 was shown to have a different transcriptional network in *K. lactis* than in *S. cerevisiae* posing the question, whether the cell related functions of the complex would be conserved between the two yeasts. In previous works it was shown that the architecture of the CWI pathway for lateral cell wall synthesis under stress is conserved between *S. cerevisiae* and *K. lactis* (Jacoby *et al.*, 1999; Kirchrath *et al.*, 2000; Rodicio *et al.*, 2006; Rodicio *et al.*, 2008b). Indeed, a similar reduction in cell wall thickness and sensitivities were found in *K. lactis* mutants lacking the subunits of the SNF1 complex. In contrast to *S. cerevisiae*, *pgi1* deletions in *K. lactis* can still grow on glucose which allowed epistasis

analyses with *Klsnf1* mutants. The results obtained with these and the *Klpfk1* and *Klpfk2* mutants were consistent with the notion that the levels of sugar phosphates may determine the compensatory capacity for cell wall synthesis. However, the most important finding of this work was that a *Klmig1* deletion also suppresses the cell wall phenotype of a *Klsnf1* deletion. This implicated the transcriptional repressor, which is a known downstream effector of SNF1 in energy signaling, to have a function in cell wall synthesis, too. Suppression by a deletion was also tested in *S. cerevisiae* strains lacking a functional SNF1 complex and a similar epigenetic relationship was discovered. It was also observed that cell wall stress decreases the levels of phosphorylated KlMig1 and therefore presumably increases its nuclear concentration and thus its repression capacity. Finally, the phosphorylation sites in KlMig1 were determined. Preliminary results indicate large overlaps with ScMig1 phosphorylation patterns, but also the existence of differentially used target sites. The identification of phosphorylation sites in KlMig1 is still in progress, which explains why the manuscript has not yet been submitted. The coverage has to be improved and different (stress) conditions need to be tested.

Further relations to sugar metabolism were also found as part of a Master thesis, whose results are not included, here. The investigation of the transcription factor Msn2 in *S. cerevisiae*, which like Msn4 is a multicopy suppressor of the *snf1* deletion phenotypes related to carbohydrate metabolism (Estruch and Carlson, 1993). Suppression was also observed for the cell wall related phenotypes of the *snf1* deletion when *MSN2* was overexpressed. Various missense mutations, especially in the C-terminus where the zinc-finger binding domain of Msn2 is located, lost their suppression capacity. However, further work is needed to find the mechanism by which Msn2 could play a role in the transcriptional activation of cell wall related genes in *S. cerevisiae*. So far Msn2 was shown in *K. lactis* to regulate mating and mating-type switching (Barsoum *et al.*, 2011). A future work could also assess the role of Nrg1, another transcriptional repressor targeted by SNF1 and shown to be important in response to high medium osmolarity (Ye *et al.*, 2008), to be addressed in both yeasts for cell wall phenotypes.

The need for cell wall synthesis at the bud neck is clearly shown in investigations of cytokinesis in *K. lactis*, when primary and secondary septa are constructed. A number of regulators for cytokinesis have been identified in *S. cerevisiae*. Although providing important hints, the central question of how these regulators relate to the synthesis and dynamics of the AMR and the PS still awaits a satisfying answer. Therefore, it was decided to investigate the process in the genetically less redundant yeast *K. lactis*, where tools for the timing and investigation of cytokinesis were not available, until now. In the manuscript Rippert *et al.* (2014) similar ultrastructure of the yeast bud neck was found for *K. lactis*, compared to data

reported for *S. cerevisiae*. Three key regulators (KlInn1, KlHof1, KlCyk3) were characterized and provided first hints on differences in the regulation compared to *S. cerevisiae* (i.e. lethality of the *Klcyk3* deletion). Furthermore, probes for life-cell microscopy were constructed, i.e. the Lifeact construct and a KlMyo1-mCherry fusion. The fact that the deletion of *Klmyo1* remains viable offers the opportunity to uncouple AMR constriction and PS formation. Another key regulator with central function in *S. cerevisiae* is Iqg1, and its ortholog KlIqg1 would be an interesting subject of future investigations.

The similarity to *S. cerevisiae* is further underlined in the second manuscript on cytokinesis (Rippert and Heinisch, 2016), which characterized septins as the basic components of the cytokinetic machinery. The five mitotic septins found are similar in their primary structure comparing each one to its ortholog in the two yeast species. All turned out to be essential for growth of a culture (with common exception of Shs1, whose lack does not have a major effect on cytokinesis). Their ability for cross-complementation hinted to a strong evolutionary conservation, up to the level of formation of heterooctameric complexes. The different septin-GFP fusions obtained in that work provide yet another tool piece to follow the timing of cytokinesis in colocalization studies with other bud neck components. In this study it was also shown that unlike KlMyo1, and in contrast to ScChs2, KlChs2 is essential indicating the central role of PS formation in cytokinesis. As in *S. cerevisiae*, *K. lactis* disposes of two more isozymes of chitin synthases (KlChs1, KlChs3), whose capacity to substitute KlChs2 functions when overproduced, is currently being investigated. Besides the disappointing high degree of similarity to *S. cerevisiae*, both studies on cytokinesis revealed one central flaw in doing molecular genetics in *K. lactis*: the lack of a conditional gene expression. Several promoters tightly regulated in *S. cerevisiae* were tested (*ScGAL1/10p*, *KlLAC4p* and *KlICL1p*), but none showed a high level of repression under the respective conditions. None of the essential genes placed under their control could be turned off sufficiently to produce the expected growth defects. This is a primary problem to focus on if *K. lactis* work is to be continued at all. The Tet-Off system, which was shown to work in the closely related *Kluyveromyces marxianus* (Pecota and Da Silva, 2004), is currently being investigated for use in *K. lactis* in a Bachelor thesis.

In summary the parallel studies of cytokinesis regulation, cell wall integrity control and energy balancing in *S. cerevisiae* and *K. lactis* revealed a surprisingly high degree of conservation given the different physiological constitution of the two yeast species, i.e. primarily fermentative *versus* respiratory metabolism. However, as demonstrated in CWI-studies and the second manuscript (Rippert *et al.*, 2016), less redundancy may be of advantage in reducing the number of genes and isozymes to be studied (one KlMkk1 *vs*. two ScMkks, one KlRom2 *vs*. two ScRoms, one KlGal83 *vs*. three Sc ß-subunits etc.). Furthermore, this may

cause yeast specific differences in importance (and presumably physiological functions) as exemplified by KlCyk3 and KlGal83. Although there is considerable amount of work ahead in terms of basic tools to study the three processes in *K. lactis* in comparison to *S. cerevisiae*, the work presented herein provides a solid basis. Given the biotechnological importance, the limited expertise on *K. lactis* as well as its genetics worldwide and the considerable amount of research and financial resources already invested, a continuation of this research would be desirable.

5. Abbreviations

℃	Degree Celcius
ADP	Adenosine diphosphate
AMP	Adenosine monophosphate
AMR	Actomyosin ring
AMPK	AMP-activated protein kinase
APC	Anaphase promoting complex
ATP	Adenosine triphosphate
bp	Base pairs
CFW	Calcofluor White
CWI	Cell wall integrity
DIC	Differential interference contrast (microscopy)
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
E. coli	*Escheria coli*
EDTA	Ethylenediaminetetraacetic acid
ER	Endoplasmic reticulum
G418	Geneticin
GDP	Guanosine diphosphate
GEF	Guanine nucleotide exchange factor
GFP	Green fluorescent protein
Glc	Glucose
GTP	Guanosine triphosphate
h	hours
HA	human influenza hemagglutinin
KAc	Potassium acetate
K. lactis	*Kluyveromyces lactis*
Kl	*Kluyveromyces lactis*
l	Liter
M	Molar
MAPK	Mitogen-activated protein kinase
MAPKK	Mitogen-activated protein kinase kinase
MAPKKK	Mitogen-activated protein kinase kinase kinase
mCh	mCherry fluorescent protein
MEN	Mitotic exit network

mg	Milligram
min	Minute
ml	Milliliter
mM	Micromolar
OD	Optical density
PAGE	Polyacrylamide gel electrophoresis
PCR	Polymerase chain reaction
PS	Primary septum
RAM	Regulation of Ace2p activity and cellular morphogenesis
s	Second
SC	synthetic complete medium
S. cerevisiae	*Saccharomyces cerevisiae*
Sc	*Saccharomyces cerevisiae*
SDS	Sodium dodecyl sulfate
SEM	Scanning electron microscopy
SH3	Sarc homology
S. pombe	*Schizosaccharomyces pombe*
SS	Secondary septa
TEM	Transmission electron microscopy
wt	Wild type
YEPD	Yeast extract peptone dextrose (rich medium)
YNB	Yeast nitrogen base
µl	Microliter
µm	Micrometer
µM	Micromolar

6. References

Amodeo, G. A., Momcilovic, M., Carlson, M. and Tong, L. (2010). Biochemical and functional studies on the regulation of the *Saccharomyces cerevisiae* AMPK homolog SNF1. *Biochem. Biophys. Res. Commun.* **397**, 197–201.

Ashrafi, K., Lin, S. S., Manchester, J. K. and Gordon, J. I. (2000). Sip2p and its partner Snf1p kinase affect aging in *S. cerevisiae*. *Genes Dev.* **14**, 1872–1885.

Backhaus, K., Heilmann, C. J., Sorgo, A. G., Purschke, G., de Koster, C. G., Klis, F. M. and Heinisch, J. J. (2010). A systematic study of the cell wall composition of *Kluyveromyces lactis*. *Yeast* **27**, 647–660.

Backhaus, K., Buchwald, U., Heppeler, N., Schmitz, H.-P., Rodicio, R. and Heinisch, J. J. (2011). Milk and sugar: Regulation of cell wall synthesis in the milk yeast *Kluyveromyces lactis*. *Eur. J. Cell Biol.* **90**, 745–750.

Baetz, K., Moffat, J., Haynes, J., Chang, M. and Andrews, B. (2001). Transcriptional coregulation by the cell integrity mitogen-activated protein kinase Slt2 and the cell cycle regulator Swi4. *Mol. Cell. Biol.* **21**, 6515–6528.

Balasubramanian, M. K., Bi, E. and Glotzer, M. (2004). Comparative analysis of cytokinesis in budding yeast, fission yeast and animal cells. *Curr. Biol.* **14**, 806–818.

Balasubramanian, M. K., Srinivasan, R., Huang, Y. and Ng, K.-H. (2012). Comparing contractile apparatus-driven cytokinesis mechanisms across kingdoms. *Cytoskeleton* **69**, 942–956.

Barsoum, E., Rajaei, N., Aström, S. U. (2011). RAS/Cyclic AMP and transciption factor Msn2 regulate mating and mating-type switching in the yeast *Kluyveromyces lactis*. *Eukaryot. Cell* **10**, 1545-1552.

Bertin, A., McMurray, M. A., Grob, P., Park, S.-S., Garcia, G., Patanwala, I., Ng, H.-L., Alber, T., Thorner, J. and Nogales, E. (2008). *Saccharomyces cerevisiae* septins: Supramolecular organization of heterooligomers and the mechanism of filament assembly. *Proc. Natl. Acad. Sci.* **105**, 8274–8279.

Bertin, A., McMurray, M. a, Pierson, J., Thai, L., McDonald, K. L., Zehr, E. a, García, G., Peters, P., Thorner, J. and Nogales, E. (2011). Three-dimensional ultrastructure of the septin filament network in *Saccharomyces cerevisiae*. *Mol. Biol. Cell* **23**, 423–32.

Bi, E. and Park, H.-O. (2012). Cell polarization and cytokinesis in budding yeast. *Genetics* **191**, 347–387.

Booth, E. A., Vane, E. W., Dovala, D. and Thorner, J. (2015). A förster resonance energy transfer (FRET)-based system provides insight into the ordered assembly of yeast septin hetero-octamers. *J. Biol. Chem.* **290**, 28388–28401.

Breunig, K. D., Bolotin-Fukuhara, M., Bianchi, M. M., Bourgarel, D., Falcone, C., Ferrero, I., Frontali, L., Goffrini, P., Krijger, J. J., Mazzoni, C., *et al.* (2000). Regulation of primary carbon metabolism in *Kluyveromyces lactis*. *Enzyme Microb. Technol.* **26**, 771–780.

Bulawa, C. E. (1993). Genetics and molecular biology of chitin synthesis in fungi. *Annu. Rev. Microbiol.* **47**, 505–534.

Cabib, E. and Schmidt, M. (2003). Chitin synthase III activity, but not the chitin ring, is required for remedial septa formation in budding yeast. *FEMS Microbiol. Lett.* **224**, 299–305.

Cabib, E., Silverman, S. J. and Shaw, J. A. (1992). Chitinase and chitin synthase 1: counterbalancing activities in cell separation of *Saccharomyces cerevisiae*. *J. Gen. Microbiol.* **138**, 97–102.

Cabib, E., Mol, P. C., Shaw, J. A. and Choi, W. J. (1993). Biosynthesis of cell wall and septum during yeast growth. *Arch. Med. Res.* **24**, 301–303.

Cassart, J. P., Georis, I., Ostling, J., Ronne, H. and Vandenhaute, J. (1995). The *MIG1* repressor from *Kluyveromyces lactis*: cloning, sequencing and functional analysis in *Saccharomyces cerevisiae*. *FEBS Lett.* **371**, 191–194.

Charbon, G., Breunig, K. D., Wattiez, R., Vandenhaute, J. and Noel-Georis, I. (2004). Key role of Ser562/661 in Snf1-dependent regulation of Cat8p in *Saccharomyces cerevisiae* and *Kluyveromyces lactis*. *Mol Cell Biol* **24**, 4083–4091.

Chen, X. J. (1996). Low- and high-copy-number shuttle vectors for replication in the budding yeast *Kluyveromyces lactis*. *Gene* **172**, 131–136.

Cid, V. J., Adamiková, L., Nombela, C., Sánchez, M. and Molina, M. (2001). Cell cycle control of septin ring dynamics in the budding yeast. *Microbiology* **147**, 1437–1450.

Cullen, P. J. and Sprague, G. F. (2000). Glucose depletion causes haploid invasive growth in yeast. *Proc. Natl. Acad. Sci. U. S. A.* **97**, 13619–13624.

DeVit, M. J., Waddle, J. A. and Johnston, M. (1997). Regulated nuclear translocation of the Mig1 glucose repressor. *Mol. Biol. Cell* **8**, 1603–1618.

Devrekanli, A., Foltman, M., Roncero, C., Sanchez-Diaz, A. and Labib, K. (2012). Inn1 and Cyk3 regulate chitin synthase during cytokinesis in budding yeasts. *J. Cell Sci.* **125**, 5453–5466.

Dong, J. and Dickson, R. C. (1997). Glucose represses the lactos-galactose regulon in *Kluyveromyces lactis* through a SNF-1 and MIG1-dependent pathway that modulates galactokinase (*GAL1*) gene expression. *Nucleic Acids Res.* **25**, 3657–3664.

Douglas, C. M., Foor, F., Marrinan, J. A., Morin, N., Nielsen, J. B., Dahl, A. M., Mazur, P., Baginsky, W., Li, W. and El-Sherbeini, M. (1994). The *Saccharomyces cerevisiae FKS1* (*ETG1*) gene encodes an integral membrane protein which is a subunit of 1,3-beta-D-glucan synthase. *Proc. Natl. Acad. Sci. U. S. A.* **91**, 12907–12911.

Estruch, F. and Carlson, M. (1993). Two homologous zinc finger genes identified by multicopy suppression in a SNF1 protein kinase mutant of *Saccharomyces cerevisiae*. *Mol. Cell. Biol.* **13**, 3872-3881.

Fang, X., Luo, J., Nishihama, R., Wloka, C., Dravis, C., Travaglia, M., Iwase, M., Vallen, E. A. and Bi, E. (2010). Biphasic targeting and cleavage furrow ingression directed by the tail of a myosin II. *J. Cell Biol.* **191**, 1333–1350.

Georis, I., Cassart, J. P., Breunig, K. D. and Vandenhaute, J. (1999). Glucose repression of the *Kluyveromyces lactis* invertase gene *KlINV1* does not require Mig1p. *Mol. Gen. Genet.* **261**, 862–870.

Georis, I., Krijger, J. J., Breunig, K. D. and Vandenhaute, J. (2000). Differences in regulation of yeast gluconeogenesis revealed by Cat8p-independent activation of *PCK1* and FBP1 genes in *Kluyveromyces lactis*. *Mol. Gen. Genet.* **264**, 193–203.

Gladfelter, A., Pringle, J. R. and Lew, D. J. (2001). The septin cortex at the yeast mother-bud neck. *Curr. Opin. Microbiol.* **4**, 681–689.

Gladfelter, A. S., Bose, I., Zyla, T. R., Bardes, E. S. G. and Lew, D. J. (2002). Septin ring assembly involves cycles of GTP loading and hydrolysis by Cdc42p. *J. Cell Biol.* **156**, 315–326.

Goffrini, P., Ficarelli, A., Donnini, C., Lodi, T., Puglisi, P. P. and Ferrero, I. (1996). *FOG1* and *FOG2* genes, required for the transcriptional activation of glucose-repressible genes of *Kluyveromyces lactis*, are homologous to *GAL83* and *SNF1* of *Saccharomyces cerevisiae*. *Curr. Genet.* **29**, 316–326.

González-Siso, M. I., Freire-Picos, M. A., Ramil, E., González-Domínguez, M., Rodríguez Torres, A. and Cerdán, M. E. (2000). Respirofermentative metabolism in *Kluyveromyces lactis*: Insights and perspectives. *Enzyme Microb. Technol.* **26**, 699–705.

Hardie, D. G. (2004). AMP-activated protein kinase: a master switch in glucose and lipid metabolism. *Rev. Endocr. Metab. Disord.* **5**, 119–125.

Hartwell, L. (1971). Genetic control of the cell division cycle in yeast. *Exp. Cell Res.* **69**, 265–276.

Hedbacker, K. and Carlson, M. (2008). SNF1/AMPK pathways in yeast. *Front. Biosci.* **13**, 2408–2420.

Heinisch, J. J., Buchwald, U., Gottschlich, A., Heppeler, N. and Rodicio, R. (2010). A tool kit for molecular genetics of *Kluyveromyces lactis* comprising a congenic strain series and a set of versatile vectors. *FEMS Yeast Res.* **10**, 333–342.

Hong, S. P. and Carlson, M. (2007). Regulation of Snf1 protein kinase in response to environmental stress. *J. Biol. Chem.* **282**, 16838–16845.

Honigberg, S. M. and Lee, R. H. (1998). Snf1 kinase connects nutritional pathways controlling meiosis in *Saccharomyces cerevisiae*. *Mol. Cell. Biol.* **18**, 4548–4555.

Huang, D., Wilson, W. A. and Roach, P. J. (1997). Glucose-6-P control of glycogen synthase phosphorylation in yeast. *J. Biol. Chem.* **272**, 22495–22501.

Inoue, S. B., Takewakt, N., Takasuka, T., Mio, T., Adachi, M., Fujii, Y., Miyamoto, C., Arisawa, M., Furuichi, Y. and Watanabe, T. (1995). Characterization and gene cloning of 1,3-beta-D-glucan synthase from *Saccharomyces cerevisiae*. *Eur. J. Biochem.* **231**, 845–854.

Iwase, M., Luo, J., Nagaraj, S., Longtine, M. S., Kim, H. B., Haarer, B. K., Caruso, C., Tong, Z., Pringle, J. R. and Bi, E. (2005). Role of a Cdc42p effector pathway in recruitment of the yeast septins to the presumptive bud site. *Mol. Biol. Cell* **17**, 1110–1125.

Iwase, M., Luo, J., Bi, E. and Toh-e, A. (2007). Shs1 plays separable roles in septin organization and cytokinesis in *Saccharomyces cerevisiae*. *Genetics* **177**, 215–229.

Jacoby, J. J., Kirchrath, L., Gengenbacher, U. and Heinisch, J. J. (1999). Characterization of KlBCK1, encoding a MAP kinase kinase kinase of *Kluyveromyces lactis*. *J. Mol. Biol.* **288**, 337–352.

Jendretzki, A., Ciklic, I., Rodicio, R., Schmitz, H.-P. and Heinisch, J. J. (2009). Cyk3 acts in actomyosin ring independent cytokinesis by recruiting Inn1 to the yeast bud neck. *Mol. Genet. Genomics* **282**, 437–451.

Jendretzki, A., Wittland, J., Wilk, S., Straede, A. and Heinisch, J. J. (2011). How do I begin? Sensing extracellular stress to maintain yeast cell wall integrity. *Eur. J. Cell Biol.* **90**, 740–744.

Jung, U. S., Sobering, A., Romeo, M. J. and Levin, D. E. (2002). Regulation of the yeast Rlm1 transcription factor by the Mpk1 cell wall integrity MAP kinase. *Mol. Microbiol.* **46**, 781–789.

Kirchrath, L., Lorberg, A., Schmitz, H.-P., Gengenbacher, U. and Heinisch, J. J. (2000). Comparative genetic and physiological studies of the MAP kinase Mpk1p from *Kluyveromyces lactis* and *Saccharomyces cerevisiae*. *J. Mol. Biol.* **300**, 743–758.

Klis, F. M., Mol, P., Hellingwerf, K. and Brul, S. (2002). Dynamics of cell wall structure in *Saccharomyces cerevisiae*. *FEMS Microbiol. Rev.* **26**, 239–256.

Ko, N., Nishihama, R., Tully, G. H., Ostapenko, D., Solomon, M. J., Morgan, D. O. and Pringle, J. R. (2007). Identification of yeast IQGAP (Iqg1p) as an anaphase-promoting-complex substrate and its role in actomyosin-ring-independent cytokinesis. *Mol. Biol. Cell* **18**, 5139–5153.

Kooistra, R., Hooykaas, P. J. J. and Steensma, H. . Y. (2004). Efficient gene targeting in *Kluyveromyces lactis*. *Yeast* **21**, 781–792.

Korinek, W. S., Bi, E., Epp, J. A., Wang, L., Ho, J. and Chant, J. (2000). Cyk3, a novel SH3-domain protein, affects cytokinesis in yeast. *Curr. Biol.* **10**, 947–950.

Kuchin, S., Vyas, V. K. and Carlson, M. (2002). Snf1 protein kinase and the repressors Nrg1 and Nrg2 regulate *FLO11*, haploid invasive growth, and diploid pseudohyphal differentiation. *Mol. Cell. Biol.* **22**, 3994–4000.

Kuilman, T., Maiolica, A., Godfrey, M., Mie Scheidel, N., Aebersold, R. and Uhlmann, F. (2015). Identification of Cdk targets that control cytokinesis. *EMBO J.* **34**, 81–96.

Lesage, G. and Bussey, H. (2006). Cell wall assembly in *Saccharomyces cerevisiae*. *Microbiol. Mol. Biol. Rev.* **70**, 317–343.

Levin, D. E. (2005). Cell wall integrity signaling in *Saccharomyces cerevisiae*. *Microbiol Mol Biol Rev* **69**, 262–291.

Levin, D. E. (2011). Regulation of cell wall biogenesis in *Saccharomyces cerevisiae*: the cell wall integrity signaling pathway. *Genetics* **189**, 1145–1175.

Lippincott, J., Shannon, K. B., Shou, W., Deshaies, R. J. and Li, R. (2001). The Tem1 small GTPase controls actomyosin and septin dynamics during cytokinesis. *J. Cell Sci.* **114**, 1379–1386.

Lodi, T., Saliola, M., Donnini, C. and Goffrini, P. (2001). Three target genes for the transcriptional activator Cat8p of *Kluyveromyces lactis*: acetyl coenzyme A synthetase genes *KlACS1* and *KlACS2* and lactate permease gene *KlJEN1*. *J Bacteriol* **183**, 5257–5261.

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

López-García, B., Gandía, M., Muñoz, A., Carmona, L. and Marcos, J. F. (2010). A genomic approach highlights common and diverse effects and determinants of susceptibility on the yeast *Saccharomyces cerevisiae* exposed to distinct antimicrobial peptides. *BMC Microbiol.* **10**, 289.

Lorberg, A., Schmitz, H.-P., Gengenbacher, U. and Heinisch, J. J. (2003). *KlROM2* encodes an essential GEF homologue in *Kluyveromyces lactis*. *Yeast* **20**, 611–624.

Lord, M., Laves, E. and Pollard, T. D. (2005). Cytokinesis depends on the motor domains of myosin-II in fission yeast but not in budding yeast. *Mol. Biol. Cell* **16**, 5346–5355.

Lutkenhaus, J., Pichoff, S. and Du, S. (2012). Bacterial cytokinesis: From Z ring to divisome. *Cytoskeleton* **69**, 778–790.

Madden, K. (1997). SBF cell cycle regulator as a target of the yeast PKC-MAP kinase pathway. *Science (80-.).* **275**, 1781–1784.

Mayer, F. V., Heath, R., Underwood, E., Sanders, M. J., Carmena, D., McCartney, R. R., Leiper, F. C., Xiao, B., Jing, C., Walker, P. a., *et al.* (2011). ADP regulates SNF1, the *Saccharomyces cerevisiae* homolog of AMP-activated protein kinase. *Cell Metab.* **14**, 707–714.

Mazur, P. and Baginsky, W. (1996). In vitro activity of 1,3-beta-D-glucan synthase requires the GTP-binding protein Rho1. *J. Biol. Chem.* **271**, 14604–14609.

McMichael, C. M. and Bednarek, S. Y. (2013). Cytoskeletal and membrane dynamics during higher plant cytokinesis. *New Phytol.* **197**, 1039–1057.

Mehlgarten, C., Krijger, J.-J., Lemnian, I., Gohr, A., Kasper, L., Diesing, A.-K., Grosse, I. and Breunig, K. D. (2015). Divergent evolution of the transcriptional network controlled by Snf1-interacting protein Sip4 in budding yeasts. *PLoS One* **10**, e0139464.

Meitinger, F., Petrova, B., Lombardi, I. M., Bertazzi, D. T., Hub, B., Zentgraf, H. and Pereira, G. (2010). Targeted localization of Inn1, Cyk3 and Chs2 by the mitotic-exit network regulates cytokinesis in budding yeast. *J. Cell Sci.* **123**, 1851–1861.

Meitinger, F., Boehm, M. E., Hofmann, A., Hub, B., Zentgraf, H., Lehmann, W. D. and Pereira, G. (2011). Phosphorylation-dependent regulation of the F-BAR protein Hof1 during cytokinesis. *Genes Dev.* **25**, 875–888.

Meitinger, F., Palani, S. and Pereira, G. (2012). The power of MEN in cytokinesis. *Cell Cycle* **11**, 219–228.

Meyer, S. (2014). Microcompartmentalization of cell wall integrity signaling in *Kluyveromyces lactis*. *Dissertation*.

Nadal, M., Garcia-Pedrajas, M. D. and Gold, S. E. (2010). The *snf1* gene of *Ustilago maydis* acts as a dual regulator of cell wall degrading enzymes. *Phytopathology* **100**, 1364–1372.

Nelson, B., Kurischko, C., Horecka, J., Mody, M., Nair, P., Pratt, L., Zougman, A., McBroom, L. D. B., Hughes, T. R., Boone, C., *et al.* (2003). RAM: A conserved signaling network that regulates Ace2p transcriptional activity and polarized morphogenesis. *Mol. Biol. Cell* **14**, 3782–3803.

Nishihama, R., Schreiter, J. H., Onishi, M., Vallen, E. A., Hanna, J., Moravcevic, K., Lippincott, M. F., Han, H., Lemmon, M. A., Pringle, J. R., *et al.* (2009). Role of Inn1 and its interactions with Hof1 and Cyk3 in promoting cleavage furrow and septum formation in *S. cerevisiae*. *J. Cell Biol.* **185**, 995–1012.

Nkosi, P. J., Targosz, B.-S., Labib, K. and Sanchez-Diaz, A. (2013). Hof1 and Rvs167 have redundant roles in actomyosin ring function during cytokinesis in budding yeast. *PLoS One* **8**, e57846.

Oh, Y., Chang, K.-J., Orlean, P., Wloka, C., Deshaies, R. and Bi, E. (2012). Mitotic exit kinase Dbf2 directly phosphorylates chitin synthase Chs2 to regulate cytokinesis in budding yeast. *Mol. Biol. Cell* **23**, 2445–2456.

ÓhÉigeartaigh, S. S., Armisén, D., Byrne, K. P. and Wolfe, K. H. (2011). Systematic discovery of unannotated genes in 11 yeast species using a database of orthologous genomic segments. *BMC Genomics* **12**, 377.

Onishi, M., Ko, N., Nishihama, R. and Pringle, J. R. (2013). Distinct roles of Rho1, Cdc42, and Cyk3 in septum formation and abscission during yeast cytokinesis. *J. Cell Biol.* **202**, 311–329.

Palani, S., Meitinger, F., Boehm, M. E., Lehmann, W. D. and Pereira, G. (2012). Cdc14-dependent dephosphorylation of Inn1 contributes to Inn1-Cyk3 complex formation. *J. Cell Sci.* **125**, 3091–3096.

Pecota, D. C. and Da Silva, N. A. (2005). Evaluation of the tetracycline promoter system for regulated gene expression in *Kluyveromyces marxianus*. *Biotechnol. Bioeng.* **92**, 117-123

Qadota, H., Python, C. P., Inoue, S. B., Arisawa, M., Anraku, Y., Zheng, Y., Watanabe, T., Levin, D. E. and Ohya, Y. (1996). Identification of yeast Rho1p GTPase as a regulatory subunit of 1,3-beta-glucan synthase. *Science (80-.).* **272**, 279–281.

Rodicio, R. and Heinisch, J. J. (2013). Yeast on the milky way: genetics, physiology and biotechnology of *Kluyveromyces lactis*. *Yeast* **30**, 165–177.

Rodicio, R., Koch, S., Schmitz, H.-P. and Heinisch, J. J. (2006). *KlRHO1* and *KlPKC1* are essential for cell integrity signalling in *Kluyveromyces lactis*. *Microbiology* **152**, 2635–2649.

Rodicio, R., López, M. L., Cuadrado, S., Cid, A. F., Redruello, B., Moreno, F., Heinisch, J. J., Hegewald, A.-K. and Breunig, K. D. (2008a). Differential control of isocitrate lyase gene transcription by non-fermentable carbon sources in the milk yeast *Kluyveromyces lactis*. *FEBS Lett.* **582**, 549–557.

Rodicio, R., Buchwald, U., Schmitz, H.-P. and Heinisch, J. J. (2008b). Dissecting sensor functions in cell wall integrity signaling in *Kluyveromyces lactis*. *Fungal Genet. Biol.* **45**, 422–435.

Rubenstein, E. M., McCartney, R. R., Zhang, C., Shokat, K. M., Shirra, M. K., Arndt, K. M. and Schmidt, M. C. (2008). Access denied: Snf1 activation loop phosphorylation is controlled by availability of the phosphorylated threonine 210 to the PP1 phospatase. *J. Biol. Chem.* **283**, 222–230.

Ruiz, A., Xu, X. and Carlson, M. (2011). Roles of two protein phosphatases, Reg1-Glc7 and Sit4, and glycogen synthesis in regulation of SNF1 protein kinase. *Proc. Natl. Acad. Sci. U. S. A.* **108**, 6349–6354.

Sanchez-Diaz, A., Marchesi, V., Murray, S., Jones, R., Pereira, G., Edmondson, R., Allen, T. and Labib, K. (2008). Inn1 couples contraction of the actomyosin ring to membrane ingression during cytokinesis in budding yeast. *Nat. Cell Biol.* **10**, 395–406.

Schaffrath, R. and Breunig, K. D. (2000). Genetics and molecular physiology of the yeast *Kluyveromyces lactis*. *Fungal Genet. Biol.* **30**, 173–190.

Schmidt, M. C. and McCartney, R. R. (2000). beta-subunits of Snf1 kinase are required for kinase function and substrate definition. *EMBO J.* **19**, 4936–4943.

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.

Schüller, H.-J. (2003). Transcriptional control of nonfermentative metabolism in the yeast *Saccharomyces cerevisiae*. *Curr. Genet.* **43**, 139–160.

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.

Smith, F. C., Davies, S. P., Wilson, W. A., Carling, D. and Hardie, D. G. (1999). The SNF1 kinase complex from *Saccharomyces cerevisiae* phosphorylates the transcriptional repressor protein Mig1p in vitro at four sites within or near regulatory domain 1. *FEBS Lett.* **453**, 219–223.

Soontorngun, N., Baramee, S., Tangsombatvichit, C. and Thepnok, P. (2012). Genome-wide location analysis reveals an important overlap between the targets of the yeast transcriptional regulators Rds2 and Adr1. *Biochem. Biophys. Res. Commun.* **423**, 632–637.

Stapleton, D., Mitchelhill, K. I., Gao, G., Widmer, J., Michell, B. J., Teh, T., House, C. M., Fernandez, C. S., Cox, T., Witters, L. a., *et al.* (1996). Mammalian AMP-activated protein kinase subfamily. *J. Biol. Chem.* **271**, 611–614.

Tonukari, N. J., Scott-Craig, J. S. and Walton, J. D. (2000). The *Cochliobolus carbonum SNF1* gene is required for cell wall-degrading enzyme expression and virulence on maize. *Plant Cell* **12**, 237–248.

Tully, G. H., Nishihama, R., Pringle, J. R. and Morgan, D. O. (2009). The anaphase-promoting complex promotes actomyosin-ring disassembly during cytokinesis in yeast. *Mol. Biol. Cell* **20**, 1201–1212.

Vallen, E. A., Caviston, J. and Bi, E. (2000). Roles of Hof1p, Bni1p, Bnr1p, and myo1p in cytokinesis in *Saccharomyces cerevisiae*. *Mol. Biol. Cell* **11**, 593–611.

VerPlank, L. and Li, R. (2005). Cell cycle-regulated trafficking of Chs2 controls actomyosin ring stability during cytokinesis. *Mol. Biol. Cell* **16**, 2529–2543.

Versele, M., Gullbrand, B., Shulewitz, M. J., Cid, V. J., Bahmanyar, S., Chen, R. E., Barth, P., Alber, T. and Thorner, J. (2004). Protein-protein interactions governing septin heteropentamer assembly and septin filament organization in *Saccharomyces cerevisiae*. *Mol. Biol. Cell* **15**, 4568–4583.

Vincent, O., Townley, R., Kuchin, S. and Carlson, M. (2001). Subcellular localization of the Snf1 kinase is regulated by specific beta subunits and a novel glucose signaling mechanism. *Genes Dev.* **15**, 1104–1114.

Watanabe, Y., Irie, K. and Matsumoto, K. (1995). Yeast *RLM1* encodes a serum response factor-like protein that may function downstream of the Mpk1 (Slt2) mitogen-activated protein kinase pathway. *Mol. Cell. Biol.* **15**, 5740–5749.

Wloka, C. and Bi, E. (2012). Mechanisms of cytokinesis in budding yeast. *Cytoskeleton* **69**, 710–726.

Wloka, C., Nishihama, R., Onishi, M., Oh, Y., Hanna, J., Pringle, J. R., Krauß, M. and Bi, E. (2011). Evidence that a septin diffusion barrier is dispensable for cytokinesis in budding yeast. *Biol. Chem.* **392**, 813–829.

Zhang, G., Kashimshetty, R., Kwee, E. N., Heng, B. T. and Foong, M. Y. (2006). Exit from mitosis triggers Chs2p transport from the endoplasmic reticulum to mother-daughter neck via the secretory pathway in budding yeast. *J. Cell Biol.* **174**, 207–220.

Zhang, J., Olsson, L. and Nielsen, J. (2010). The beta-subunits of the Snf1 kinase in *Saccharomyces cerevisiae*, Gal83 and Sip2, but not Sip1, are redundant in glucose derepression and regulation of sterol biosynthesis. *Mol Microbiol* **77**, 371–383.

Zhao, C., Jung, U. S., Garrett-Engele, P., Roe, T., Cyert, M. S. and Levin, D. E. (1998). Temperature-induced expression of yeast FKS2 is under the dual control of protein kinase C and calcineurin. *Mol. Cell. Biol.* **18**, 1013–1022.

Zimmermann, F. K., Kaufmann, I., Rasenberger, H. and Haußmann, P. (1977). Genetics of carbon catabolite repression in *Saccharomyces cerevisiae*: genes involved in the derepression process. *Mol. Gen. Genet.* **151**, 95–103.

7. Acknowledgements

An dieser Stelle möchte ich mich bei allen bedanken, die mir während meiner Doktorarbeit beigestanden sowie in vielfältiger Art und Weise geholfen haben.

Ein besonderer Dank gilt Prof. Dr. Jürgen Heinisch für die Ermöglichung dieser Dissertation, für die hilfreiche Unterstützung bei der Erstellung und für die Hilfs- und Diskussionsbereitschaft.

Herrn Prof. Dr. Hans Merzendorfer danke ich herzlich für die Übernahme des Zweitgutachtens.

Des Weiteren danke ich Herrn PD Dr. Hans-Peter Schmitz für die stete Hilfsbereitschaft bei technischen Problemen und Fragestellungen und für die Teilnahme an der Prüfungskommission.

Bei Dr. Stefan Walter möchte ich mich für die bereitwillige Teilnahme an der Prüfungskommission bedanken sowie für die Hilfe bei der massenspektrometrischen Bestimmung von Phosphorylierungsstellen.

Bedanken möchte ich mich auch bei Herrn apl. Prof. Dr. Günter Purschke, dass er mir die Benutzung des Elektronenmikroskops ermöglicht hat. Des Weiteren geht ein Dank an Werner Mangerich, der mir die Zellfixierung und die Benutzung des Rasterelektronenmikroskops gezeigt hat.

Bei der ganzen Arbeitsgruppe Genetik bedanke ich mich hiermit für eine tolle Zeit und für die positive Arbeitsatmosphäre. Bernadette gilt außerdem ein großer Dank für sehr viele hilfreiche Ratschläge und die Unterstützung bei den Western Blots und der Proteinaufreinigung. Bei Andrea möchte ich mich für die Hilfe bei der Herstellung der Metabolite bedanken.

Bei Anne und Bernadette möchte ich mich noch besonders für die schöne Zeit bedanken, die wir gemeinsam im Labor 329 verbracht haben.

Als letztes möchte ich mich noch bei meiner Familie bedanken, die immer hinter mir steht und mich unterstützt. Ein besonderer Dank gilt meiner Schwester Martina, die meine Arbeit Korrektur gelesen und mir Ratschläge gegeben hat.

8. Statutory declaration

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 Quellen gekennzeichnet.

Eigenleistung an der Studie: „Mutations in SNF1 complex genes affect yeast cell wall strength“ (Kapitel 3.1):

- 40 % Anteil an der Durchführung der Experimente für das Manuskript.

Eigenleistung an der Studie: „Mig1 mediates regulation of cell wall synthesis by the SNF1 kinase complex in *Kluyveromyces lactis*“ (Kapitel 3.2):

- 90 % Anteil an der Durchführung der Experimente für das Manuskript.

Eigenleistung an der Studie: „Regulation of cytokinesis in the milk yeast *Kluyveromyces lactis*“ (Kapitel 3.3):

- 50 % Anteil an der Durchführung der Experimente für das Manuskript.

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

Mithilfe an der Studie: „Four mitotic septins and chitin synthase 2 are essential for cytokinesis in *Kluyveromyces lactis*“ (Kapitel 3.4):

- Frau Julia Nagel half im Rahmen ihrer Bachelorarbeit bei der Konstruktion der Stämme KJN1 (*KlSHS1/Klshs1::ScURA3*), KJN3 (*KlCDC10/Klcdc10::ScUA3*), KJN4 (*KlCDC12/Klcdc12::ScLEU2*), KJN5 (*KlSHS1/Klshs1::ScURA3 KlMYO1/KlMYO1-mCherry*) sowie Segreganten daraus, und der Plasmide pJN3 (p*KlCDC10*), pJN4 (p*KlSHS1*), pJN5 (p*KlCDC11*) und pJN6 (p*KlCDC12*).
- Frau Kareen Veldmann half im Rahmen ihrer Bachelorarbeit bei der Konstruktion der Stämme KKV3 (*KlCHS2/Klchs2::ScLEU2*), BKV1 (*ScCDC3/Sccdc3::kanMX*), BKV2 (*ScCDC11/Sccdc11::kanMX*) und BKV3 (*ScCDC12/Sccdc12::kanMX*), und der Plasmide pKV5 (p*ScCDC10*), pKV6 (p*ScCDC10*), pKV11 (p*ScSHS1*) und pKV13 (p*KlCDC10*).

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.

_____ _____

(Ort, Datum) (Unterschrift)

9. Curriculum vitae

Name: Dorthe Rippert
Geburtsdatum: 28.02.1987
Staatsangehörigkeit: deutsch

Bildungsweg:

07/1999 – 06/2006	Gymnasium Ulricianum Aurich Abschluss: Abitur
10/2006 – 09/2009	Universität Osnabrück, Fachbereich Biologie/Chemie Bachelor of Science Biologie der Zellen Abschluss: Bachelor of Science
10/2009 – 11/2011	Universität Osnabrück, Fachbereich Biologie/Chemie Master of Science Biologie der Zellen Abschluss: Master of Science
12/2011 – 06/2016	Universität Osnabrück, Fachbereich Biologie/Chemie Promotion Biologie, Abt. Genetik Abschluss: Dr. rer. nat.