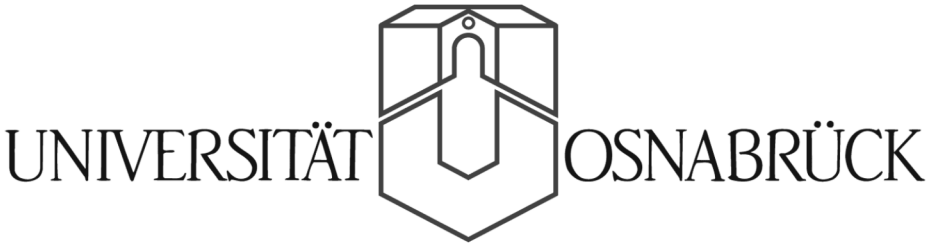

Comparative analyses of cell wall integrity signaling and cytokinesis regulation in the yeasts *Saccharomyces cerevisiae* and *Kluyveromyces lactis*

Dissertation

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vorgelegt von

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1 Introduction

The ability to sense and respond to environmental changes is probably one of the most ancient inventions in cellular evolution. Thus, from single-cell microorganisms to mammalian cells embedded in their tissues, signaling pathways operate to elicit the appropriate cellular responses to physical and chemical variations in the extracellular medium, such as temperature shifts, variations in medium pH, the presence of nutrients, hormones or potentially harmful compounds etc. In eukaryotes, such signaling pathways frequently involve membrane spanning sensors as the connective elements between environmental clues and their conversion to intracellular biochemical transduction chains. In many cases, the sensors trigger the activation of small G-proteins, which are then coupled through a limited number of mediator proteins to so-called mitogen-activated protein kinase (MAPK) cascades. The latter consist of a highly conserved three-partite set of protein kinases (MAPKKK, MAPKK, and MAPK, for MAP kinase kinase kinase etc.), which each are activated by phosphorylation of the proceding kinase. The MAPK at the lower end of this cascade may regulate the activity of transcription factors and/or that of other target proteins by phosphorylation, which then triggers the proper transcriptional and physiological responses.

In the yeast *Saccharomyces cerevisiae* at least five such MAPK pathways have been described, which mediate the response to mating pheromones, to nitrogen limitation, to either high or low medium osmolarity, and to conditions provoking sporulation (reviewed in Hohmann, 2002). Amongst them, the cell wall integrity (CWI) pathway mediates the response to hyperosmotic conditions and triggers new cell wall synthesis upon various stresses (reviewed in Heinisch et al., 1999; and in Levin, 2005). This pathway, which constitutes one of the research focuses in our laboratory, is depicted in Fig. 1.1.

1.1 The CWI pathway of *S. cerevisiae*

The membrane-spanning sensors Wsc1 and Mid2 are generally regarded as the key upstream components of the CWI pathway in *S. cerevisiae* (Levin, 2005). Yet, they each belong to a small subfamily of CWI sensors also comprising Wsc2 and Wsc3, as well as Mtl1. Due to the very subtle phenotypes caused by deletions of the genes

encoding the latter, the importance of these sensors is still obscure (reviewed in Rodicio and Heinisch, 2010, and in Jendretzki et al., 2011). The major sensors Wsc1 and Mid2 stimulate the signaling pathway upon cell wall stress by recruiting, and presumably activating, the GEF Rom2 (Philip and Levin, 2001), which in turn activates the small GTPase Rho1.

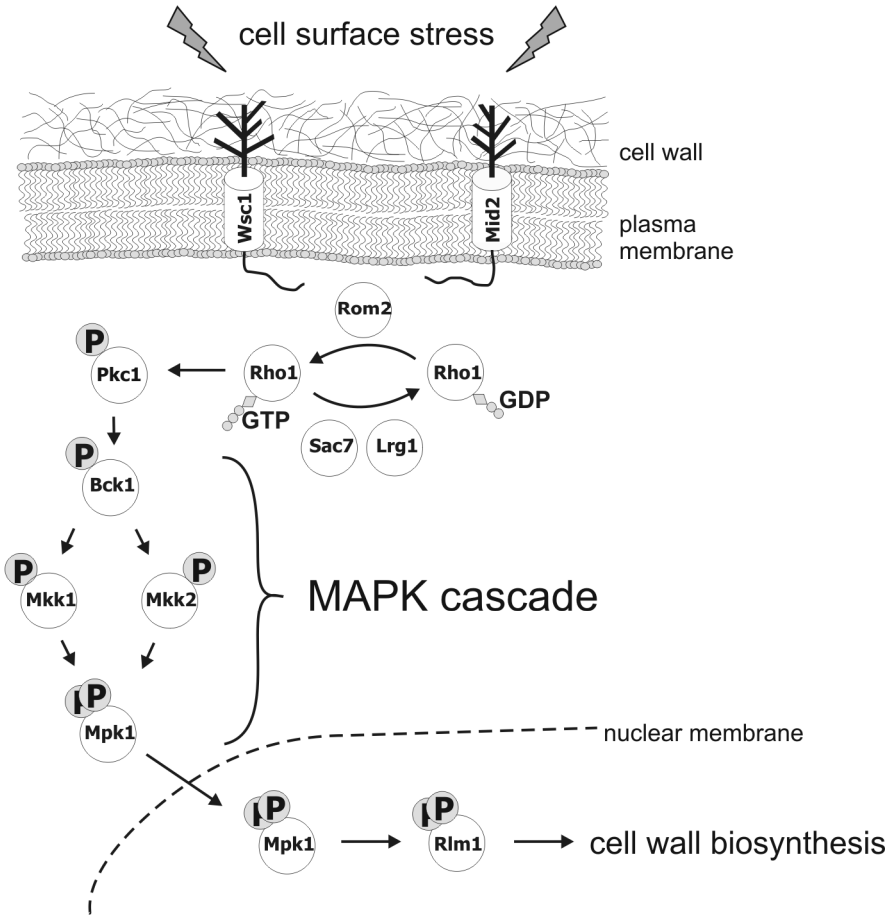


Fig. 1.1: Schematic representation of the CWI signal transduction pathway
Stress on either the cell wall or on the plasma membrane is detected by the two main sensors Mid2 and Wsc1 which interact with the GDP/GTP exchange factor Rom2. This catalyzes the conversion of Rho1 to its GTP-bound (active) state, which in turn activates Pkc1. This upstream kinase triggers the MAPK cascade (Bck1, Mkk1/Mkk2 and Mpk1). The activated MAPK Mpk1 phosphorylates the transcription factor Rlm1, which then up-regulates cell wall biosynthesis.

Wsc1 has been shown to possess the mechanical properties of a nanospring and therefore may act as a mechanosensor (Dupres et al., 2009). Moreover, it tends to cluster under stress conditions, which has been proposed to enhance the signal strength by the formation of a “Wsc1 sensosome” (Heinisch et al., 2010b; reviewed in Heinisch and Dufrene, 2010).

The active, GTP-bound Rho1 interacts with the sole yeast protein kinase C (Pkc1) through the HR1A and C1B domains (Schmitz et al., 2002; reviewed in Schmitz and Heinisch, 2003). Phosphorylation by Pkc1 presumably activates the MAPKKK Bck1 and thereby the entire MAP-cascade, besides Bck1 comprising the redundant

MAPKKs Mkk1 and Mkk2, as well as the MAPK Mpk1, which becomes dually phosphorylated at a threonine and a tyrosine residue within the conserved TEY target sequence (Martin et al., 2000; Jacoby et al., 1999). Mpk1 then phosphorylates its target proteins, of which the transcription factor Rlm1 has been shown to activate the expression of 25 genes, whose products either constitute cell wall components or enzymes involved in cell wall biosynthesis (Jung and Levin, 1999).

1.2 Down-regulation of the CWI pathway

From the CWI pathway as depicted in Fig. 1.1, the activation upon cell surface stress has been well studied. On the other hand, down-regulation of the pathway should be achieved by phosphatases, which counteract the activations triggered by the various kinases, as well as by the activity of the GTPase activating proteins (GAPs), which act on Rho1 (i.e. Sac7 and Lrg1 as the major players; Lorberg et al., 2001). Four protein phosphatases have been described so far to act on components of the CWI pathway, which can be divided into two subgroups: the tyrosine phosphatases and the dual-specificity phosphatases (DSP). The protein tyrosine phosphatases Ptp2 and Ptp3 act on the phosphorylated tyrosine within the activation loop of Mpk1, but also on similar residues within MAPKs involved in other signal transduction pathways (reviewed in Martin et al., 2005). While these negative regulators localize either exclusively to the nucleus (Ptp2) or within the cytoplasm (Ptp3) (Mattison et al., 1999), the DSPs Msg5 and Sdp1 are found in both compartments. Due to their shallow phosphate-binding pocket, the latter can remove phosphate groups not only from the tyrosine residue, but also from serines and threonines of the dually phosphorylated MAPKs (Denu and Dixon, 1998). In contrast to Ptp2, Ptp3 and Msg1, Sdp1 seems to be highly specific for Mpk1, since interaction analyses revealed, that Sdp1 only acts on this MAPK and not on those of the other pathways, like Hog1, Fus3 or Kss1 (Collister et al., 2002). Also in contrast to the other mentioned phosphatase, Sdp1 apparently does not regulate the basal activity of Mpk1, but acts exclusively upon heat stress (Hahn and Thiele, 2002).

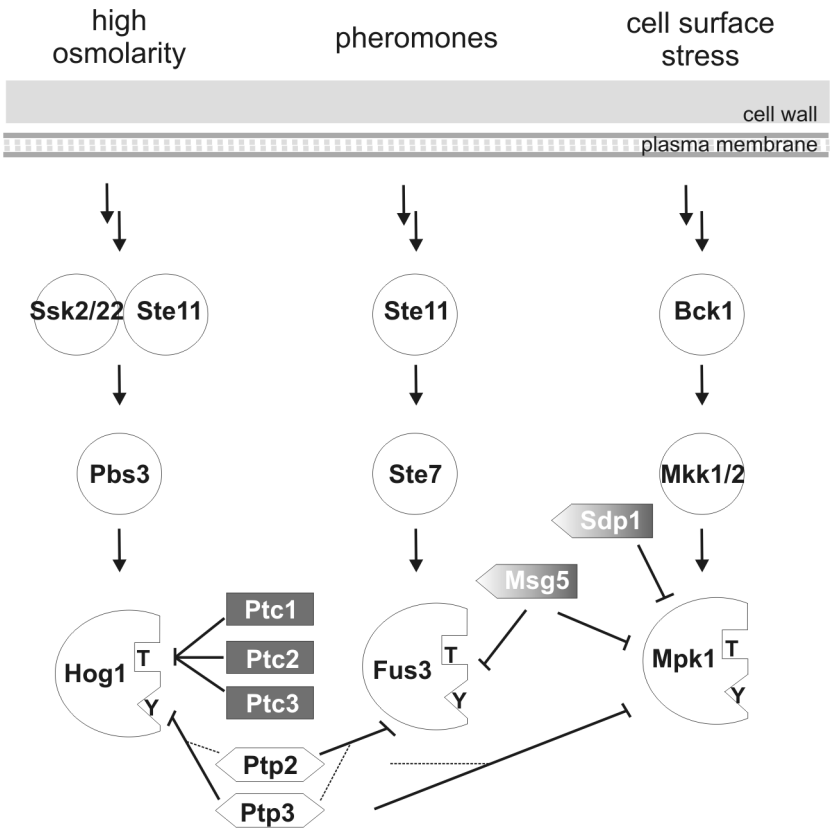


Fig. 1.2: Schematic diagram of down-regulation of yeast MAPK cascades (modified from Martin et al., 2005)
High osmolarity, pheromones and cell wall damage lead to the activation of Hog1, Fus1 and Mpk1 MAPK pathways, respectively, by phosphorylation of conserved tyrosine and threonine in the activation loop. Serin/threonine phosphatases (dark grey), tyrosine phosphatases (light grey) and dual-specificity phosphatases (light to dark grey) are also differentiated by form in the figure, according to their specificity. Thick arrows indicate the major dephosphorylating role while thinner arrows indicate minor effects (modified from Martin et al., 2005).

1.2.1 A genetic screen for putative negative regulators of the CWI pathway

Since the phosphatases apart from Sdp1 seem to act rather unspecifically on various MAPKs, a genetic screen has been established in our group to look for yet unidentified specific negative regulators of the CWI pathway (Fig. 1.2; PhD thesis of H.-P. Schmitz). In brief, the screen is based on the properties of Rlm1, which upon phosphorylation by Mpk1 can activate the transcription of its target genes (Jung and Levin, 1999). Its DNA-binding domain was substituted in a chimeric fusion protein by the bacterial lexA-binding domain. Moreover, a *lacZ*-fusion construct, whose expression is governed by a promoter containing such lexA binding motifs, was obtained. It constitutively binds the fusion protein and activates *lacZ*-transcription only upon being phosphorylated by Mpk1. This system, placed on a centromeric yeast vector, thus serves as an indirect readout of CWI pathway by determination of the associated ß-galactosidase activities.

Mutation of a gene encoding a putative negative regulator of CWI pathway activity should then result in an increase in specific ß-galactosidase activities in strains carrying this reporter construct. This can be monitored by the intensity of the blue color developing on agar plates containing X-Gal (reviewed in Heinisch, 2005). Thus, transposon-insertion mutants giving rise to dark blue colonies were analyzed for the associated gene disruption, i.e. affected in a putative negative regulator of the CWI pathway. In a first screen performed under normal growth conditions (i.e. at 30℃ in the absence of osmotic stabilization by 1M sorbitol), 27 mutants were identified (PhD thesis of H.-P. Schmitz). In the first part of this Ph.D. thesis, three of those genes were further characterized.

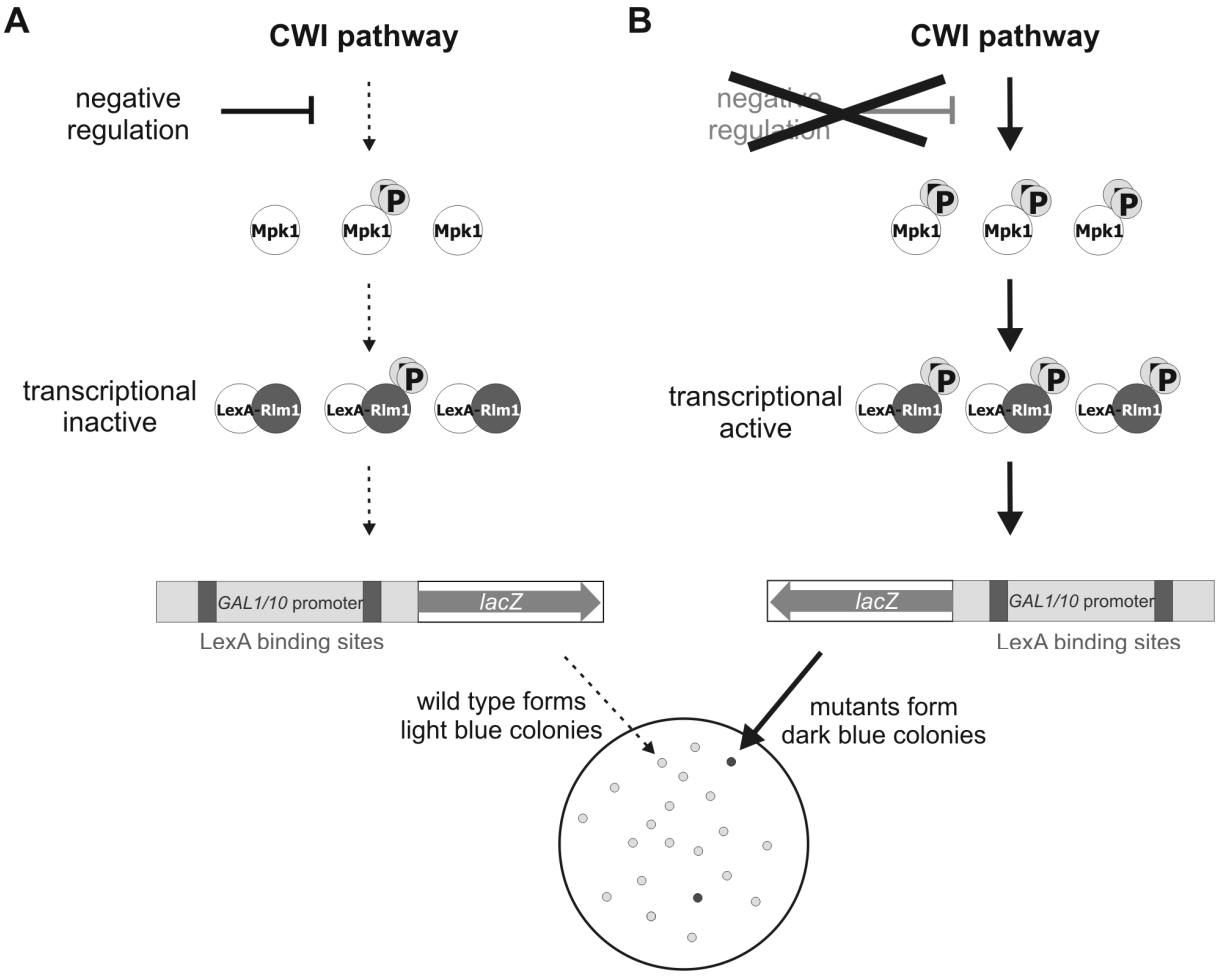


Fig. 1.2: Scheme of the *lexA-RLM1*-reporter strain
A: In wild-type cells, a basal level of the fusion protein LexA-Rlm1 is phosphorylated with a concomitant weak expression of the *lacZ* reporter gene, resulting in light blue colonies. **B:** In cells with a defect in negative regulation the higher level of activated LexA-Rlm1 leads to a stronger *lacZ*-expression, which results in the formation of dark blue colonies.

1.2.2 The putative negative regulators Nta1, Set4 and Fig4

One of the three genes which were affected by the transposon mutagenesis and were chosen for further analysis of their influence on the CWI stress response was *NTA1*, whose product has been suggested to be a N-terminal amidase. These enzymes remove the amide group from N-terminal asparagine and glutamine residues of their target proteins, converting them either to aspartate or to glutamate, respectively (Baker and Varshavsky, 1995). The latter then become targets for ubiquitin-mediated degradation according to the “N-end rule” (Varshavsky et al., 1987). Thus, Nta1 regulates the degradation, and thereby the *in vivo*-activity, of specific target proteins and was suspected to control the turnover of one or more CWI pathway components, explaining its appearance in the genetic screen.

The second candidate to be studied in further detail for its possible role in CWI signaling is encoded by the yet uncharacterized open reading frame *YJL105w.* Alignments of its deduced amino acid sequence suggested that it contains a PHD finger (**P**lant **H**omeo **D**omain) and a so-called SET domain (**S**uvar 3-9 [**Su**ppressor of position effect **var**iegation], **E**nhancer-of-zeste, **T**rithorax, Tschiersh et al., 1994), leading to its abbreviation as *SET4*. Null mutants were shown to display an increased competitive fitness in relation to wild-type, and overexpression of the gene causes vegetative growth defects, as well as a decreased resistance to rapamycin. Since proteins of the SET family are known to be involved in transcriptional regulation (Pijnappel et al., 2001), Set4 could well influence expression of one or more genes involved in cell wall construction.

Finally, *FIG4* has been described as encoding a phosphatidylinositol-3,5-bisphosphate-phosphatase, whose expression is induced by mating pheromones and upon hyperosmotic shock (Rudge et al., 2004). Fig4 forms a complex with Vac14 and gene deletion mutants display defects in shmoo formation during mating, indicating that the protein is somehow involved in determination of the cell shape (Erdman et al., 1998). A lack of Fig4 is suspected to alter the phosphorylation state of phosphoinositides as key components of cellular membranes, which may well affect CWI signaling, since it is not only activated upon stress on the cell wall itself, but also by compounds destabilizing the plasma membrane, such as chlorpromazine or tea tree oil (Straede et al., 2007).

1.3 Regulation of cell cycle and cytokinesis in *S. cerevisiae*

Considering the basic biological processes involved in yeast proliferation, it is to be expected that cell wall biosynthesis and the CWI pathway are intimately connected to the regulation of the cell cycle. Thus, the formation of new buds, as well as the separation of daughter from mother cells, requires massive synthesis of cell wall components and their constant remodeling, respectively. It is therefore not surprising, that another ORF identified in the above mentioned genetic screen, encodes the Inn1 protein (named after its proposed role in plasma membrane ingression), which was subsequently shown to be a key regulator of cytokinesis (Jendretzki et al., 2009; Sanchez-Diaz et al., 2008). This finding supports the existence of a common regulatory circuit, which coordinates cell cycle progression and cell wall biosynthesis, and opened up a new line of research for the genetics department in Osnabrück. In the following, the comparatively well-studied regulation of cytokinesis in *S. cerevisiae* will be described, as a basis for the investigation of these processes in the milk yeast *Kluyveromyces lactis*, which is the primary subject of this thesis. Cytokinesis is defined as the process when the cytoplasmata of mother and new daughter cell are separated. In yeasts, it is usually followed by the separation of mother and daughter cells. Yeast cytokinesis therefore requires the formation of a septum built up of cell wall material, as well as the remodeling of the cell wall during septum formation and prior to cell separation.

Cytokinesis can also be regarded as the last step of the cell cycle, whose progression is basically regulated by the action of a cyclin-dependent kinase (Cdk) in a dimeric complex with certain cyclins (Lew and Reed, 1993). The individual phases and the control of cell cycle progression are conserved in all eukaryotes, from yeast to humans. Thus, the cell division cycle can be divided into two major phases, namely mitosis and interphase, during which DNA replication, cell growth and metabolism occur. The interphase can be subdivided into three further phases, namely the G1-, S- and G2-phase, where “G” designates cytological “gaps” with little intracellular events distinguishable by microscopic examinations, and “S” designates the time of nuclear DNA replication. In yeast, these phases can be related to distinct morphological hallmarks (Fig. 1.3). Thus, the smaller daughter cell resulting from cytokinesis resides in G1 phase and undergoes isotropic growth. The transition into S-phase is then marked by bud emergence. In *S. cerevisiae*, the G2 phase is hardly distinguishable and may be very short under optimal growth conditions. In mitosis the

spindle apparatus is formed, chromosomes are segregated and nuclear division occurs. This phase is terminated by cytokinesis and after cell separation the next round of cell cycle occurs, provided that nutrients and other environmental cues are abundant.

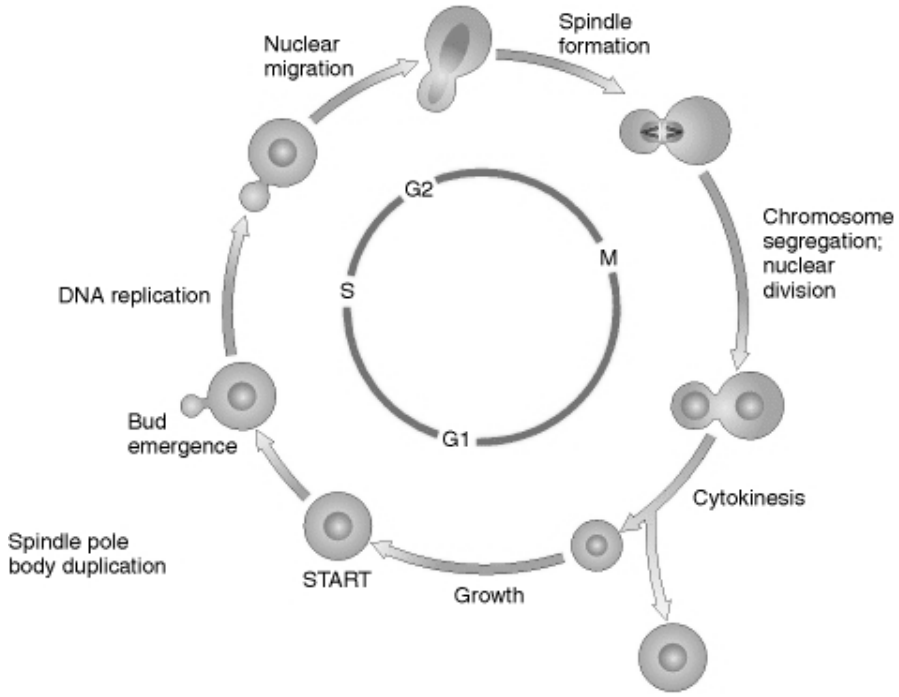


Fig. 1.3: The cell division cycle in yeast (modified from Griffiths et al., 1999)
The cell cycle can be divided into two phases: mitosis (M) and interphase, which is subdivided into G1-, S- and G2-phase. Intracellular events are related to distinct phases as hallmarks.

As mentioned above, the new bud emerges during the transition from G1- to S-phase. This is accompanied by the appearance of hour glass shaped structures, whose location is determined by septins, which begin to accumulate underneath the plasma membrane at the collar of the new bud neck (Fig. 1.4). The septins serve as scaffold for the assembly of a contractile actomyosin-ring (CAR), as well as a diffusion barrier for further proteins, which are recruited to this microcompartment. Amongst the latter are regulatory factors, which control the subsequent steps of cytokinesis, and which are described in more detail below. Towards the end of mitosis, Myo1, the only type-II-myosin in *S. cerevisiae*, is recruited to the bud neck and assembles together with F-actin in a ring-like structure along the septin-ring, building the CAR. The latter also contains other components, such as adaptor proteins, which mediate the recruitment of further regulators.

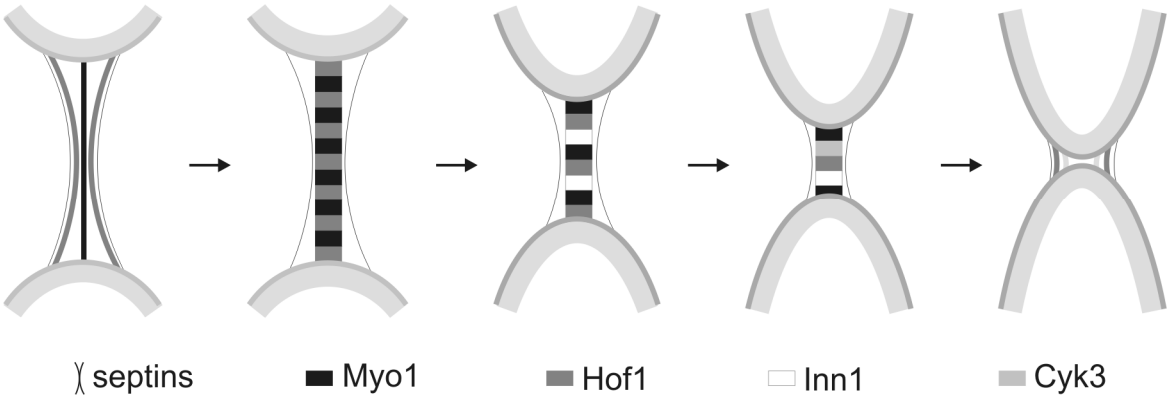


Fig. 1.4: Schematic drawing of cytokinesis in *S. cerevisiae* (modified from PhD thesis of Jendretzki, 2010)
In a cross cut section ingression of the plasma membrane in the bud neck region during cytokinesis is shown. In the first step, the septin collar is assembled (thin lines). Next, myosin II (black solid line) accumulates as a ring on the septin scaffold. Actin and other proteins are incorporated into the myosin ring to form the functional CAR (dashed line). Additionally, chitin synthase II (dark gray dot) is delivered to the bud neck. In the fourth step, constriction of the CAR pulls in the plasma membrane, which is accompanied by the synthesis of the primary chitinous septum (dark gray line). Finally, abscission of the cytoplasmata completes cytokinesis and the primary septum is closed.

Morphologically, the CAR constriction is first accompanied by the formation of a primary septum. This is made up of chitin and its synthesis has been shown to be catalyzed by the chitin synthase II isoform of *S. cerevisiae,* encoded by *CHS2*). After ring constriction and abscission of the plasma membranes, a secondary septum predominantly consisting of glucan chains and several cell wall proteins is deposited onto this primary septum. A controlled partial degradation of this secondary septum from the site of the daughter cell finally allows cell separation (reviewed in (Roncero and Sanchez, 2010).

Regarding the constriction of the CAR, and therefore of the entire cytokinesis, a set of specific regulators has been described in *S. cerevisiae* (Fig. 1.5): At the beginning of cytokinesis and shortly after recruitment of Myo1 to the CAR, Hof1 appears at the division site and forms two discs, one each at the mother and the daughter cell site of the bud neck. With the beginning of ring constriction, Hof1 relocates to the CAR and forms a single disc. This follows the constriction and upon its completion finally relocates to the septin collar and degradation takes place (Lippincott and Li, 1998). Besides Hof1, Inn1 and Cyk3 are also recruited to the bud neck during CAR constriction, although appearing much later and residing for a shorter period during cytokinesis (Jendretzki et al., 2009). Hof1 and Cyk3 seem to have somewhat redundant functions in the regulation of cytokinesis in *S. cerevisiae,* since mutants lacking either of these proteins are viable, but double deletions in the encoding genes are not. In this context it should be noted, that apparently two pathways allow for cytokinesis to occur in *S. cerevisiae.* Cells defective in one of the CAR components

are still viable in some genetic backgrounds and appear to force cytokinesis by an excessive synthesis of glucan at the putative bud neck (Bi et al., 1998).

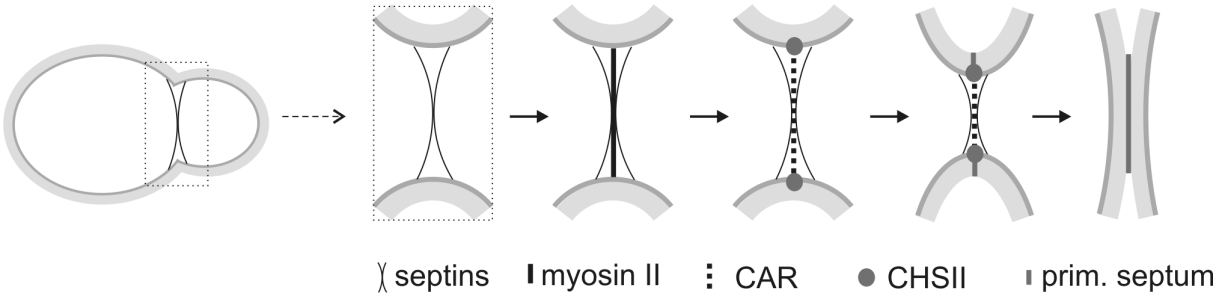


Fig. 1.5: Schematic drawing of regulatory components of cytokinesis in *S. cerevisiae*
At the beginning of cytokinesis, Myo1 (black) is recruited to the bud neck. At this stage, Hof1 (dark gray) forms two discs from both sides of the bud neck, where the septins (thin black lines) also form such an hour glass shaped structure. At the onset of cytokinesis Hof1 then relocates to the contractile ring, which also recruits Inn1 (white) and Cyk3 (light gray) while the ring is contracting. Inn1 is believed to drag the plasma membrane along, with its ingression and final abscission completing the separation of the cytoplasmata of mother and daughter cell.

Nevertheless, the newly identified *INN1* gene seems to be essential for both routes of cytokinesis, since *inn1* deletions are not viable in most *S. cerevisiae* strains (Jendretzki et al., 2009). Barely viable segregants obtained in another genetic background are probably due to one or more yet unidentified suppressor mutations (Nishihama et al., 2009). Genetic and biochemical analyses indicate, that Inn1 interacts with both Hof1 and Cyk3 through their respective SH3-domains, which recognize a number of proline-rich sequences (PRMs; “PXXP” motifs) located in the C-terminal half of Inn1 (Ito et al., 2001; Jendretzki et al., 2009). It has been proposed that Hof1 and Cyk3 act consecutively to recruit Inn1 to the plasma membrane at the bud neck and that Inn1 may be essential for the final abscission event (Jendretzki et al., 2009). However, neither has this latter function been demonstrated experimentally yet, nor is it clear, if this essential role of Inn1 in cytokinesis can be extended beyond the cell division in *S. cerevisiae.* Thus, the homolog of the fission yeast, *Schizosaccharomyces pombe,* called Fic1, which has been investigated so far, has rather little influence on cytokinesis, as judged from the lack of phenotypes in deletion mutants (Roberts-Galbraith et al., 2009). However, it should be noted that septum formation in *S. pombe* does not involve chitin synthesis and may thus not be representative for the majority of yeast species (reviewed in Roncero and Sanchez, 2010).

In *K. lactis* none of the above mentioned components has been studied until now.

1.4 Biology and genetics of the milk yeast *Kluyveromyces lactis*

Within the last two decades, *K. lactis* has been established as an alternative and powerful model organism besides the budding yeast *S. cerevisiae*. A key feature related to its name is the ability to utilize lactose as a carbon source, due to the presence of a lactose permease and an intracellular ß-galactosidase (Webster and Dickson, 1988). In dairy industries, it is frequently employed to remove the disaccharide, e.g. for the sake of lactose-intolerant patients (Breunig et al., 2000; Snoek and Steensma, 2006).

Like *S. cerevisiae K. lactis* belongs to the group of endoascomycetous yeasts. It disposes of two mating types (a and alpha), proliferates by budding in the haploid and in the diploid state (although the latter is somewhat less stable than in *S. cerevisiae*; Kooistra et al., 2004), and is therefore amenable to classical genetics by crossing, sporulation and tetrad analysis (Schaffrath and Breunig, 2000). Recently, a congenic *K. lactis* series has been obtained, based on the strain used for the whole genome sequencing, which provides the opportunity for manipulations in a relatively constant genetic background (Heinisch et al., 2010a). Moreover, vector systems already existing for various purposes (Chen, 1996) were considerably improved, and the tools for the construction and investigation of specific deletion mutants by homologous recombination, similar to those employed in *S. cerevisiae*, are available (Heinisch et al., 2010a). Yet, the efficiency of homologous recombination in *K. lactis* is much lower than in *S. cerevisiae*, frequently impeding the use of one-step gene deletion procedures with PCR products comprising only 40 bp of homology to the target sequences at each end, as routinely employed in *S. cerevisiae* (Longtine et al., 1998). Rather, illegitimate recombination occurs in *K. lactis*. This problem can be at least partially overcome by the use of *ku80* null mutants, which eliminate the pathway for non-homologous end joining (Kooistra et al., 2004; Heinisch et al., 2010).

Regarding energy metabolism, *S. cerevisiae* displays the Crabtree effect and mainly produces alcohol from high-sugar contents even in the presence of oxygen. In contrast, *K. lactis* shows the Pasteur Effect and relies on a predominantly aerobic metabolism, which gains energy predominantly from respiration (Tarrio et al., 2006).

From an evolutionary point of view, both *K. lactis* and *S. cerevisiae* share a common progenitor with a haploid genome (Kellis et al., 2004). Sequence analyses showed that a whole genome duplication (WGD) occurred later on in *S. cerevisiae* and gave

rise to the 16 chromosomes of the current haploid genome, with comparatively high redundancy in their genetic information. The genome of *K. lactis* did not undergo such a duplication and comprises ~12 Mbp distributed on six chromosomes (A-F) in the haploid genome (Dujon et al., 2004). This makes comparative studies in *K. lactis* especially attractive, since phenotypes can be frequently observed in single gene deletions, whereas in *S. cerevisiae* two or more homologues need to be knocked out due to their functional redundancy (see for example Lorberg et al., 2003).

Since the scientific community working on *K. lactis* is comparatively small, major regulatory networks, which are well characterized in *S. cerevisiae*, have barely been tackled. One of the signal transduction routes studied in some depth in *K. lactis* is the CWI pathway (reviewed in Backhaus et al., 2011). Despite several peculiarities and a lower redundancy in its components, it seems to follow more or less the order of events depicted in Fig. 1.1 for *S. cerevisiae*. Briefly, *K. lactis* disposes of three membrane-spanning sensors, *Kl*Wsc1, *Kl*Wsc2/3, and *Kl*Mid2 (Rodicio et al., 2008), which signal to *Kl*Rom2, the single and essential GEF of the pathway (Lorberg et al., 2003). *Kl*Rho1 and *Kl*Pkc1 show similar yeast two-hybrid (YTH) interactions as do their *S. cerevisiae* counterparts and even display heterologous interactions amongst the two yeast species (Rodicio et al., 2006). Deletions in *KlBCK1*, encoding the MAPKKK, display much weaker phenotypes as compared to *Scbck1* null mutants (Jacoby et al., 1999). *Klmpk1* deletions, which lack the downstream MAPK of the module, are sensitive to various cell wall stresses, again reminiscent of the respective *S. cerevisiae* null mutants (Kirchrath et al., 2000). Unpublished data from our group indicate that *KlMKK1* encodes the sole MAPKK of *K. lactis*, is not essential, but displays CWI phenotypes as expected (e.g. sensitivities to Calcofluor white and Congo Red; J. J. Heinisch, personal communication).

Besides these studies, the MAPK module of the pheromone pathway of *K. lactis* has been investigated in some detail (Kawasaki et al., 2008). Moreover, the high osmolarity glycerol (HOG) pathway also seems to serve similar functions in *K. lactis* as it does in *S. cerevisiae*, although the experimental data are still relatively scarce (Siderius et al., 2000).

Regarding the control of cytokinesis, *K. lactis* has not been studied at all, until now, with relation to the molecular mechanisms and pathway components described in chapter 1.3 for *S. cerevisiae*. Therefore, these investigations constitute a major part of this thesis.

1.5 Aims of the thesis

The basic biological process to be addressed in this thesis was the regulatory cross-talk between the pathways governing cell wall biosynthesis (i.e. the CWI pathway) on one hand, and those controlling yeast cytokinesis on the other hand. To this end, the aims of the thesis can be divided into two unequal parts:

1. The role of the putative negative regulators of the CWI pathway described above (Nta1, Set4 and Fig4), which were isolated in a genetic screen in *S. cerevisiae*, was to be further investigated. This part of the thesis presents rather preliminary data and was predominantly designed to narrow down the components presenting a regulatory link between CWI signaling and cytokinesis. No specific involvement in the CWI pathway has been assigned to any of these three proteins, so far.

2. The major part of the thesis focused on the investigation of cytokinesis in *K. lactis.* As described in chapter 1.3, despite of rather intensive research performed on the regulation of cytokinesis in *S. cerevisiae* over the past decades, it was only recently that a new important component was identified with Inn1 by our group and others (Jendretzki et al., 2009; Sanchez-Diaz et al., 2008). Although *Sc*Inn1 has been proposed to mediate plasma membrane ingression during cytokinesis, the exact molecular mechanism of its action and its interplay with two other key regulators, *Sc*Hof1 and *Sc*Cyk3, remains obscure. Since homologues of all three genes encoding these proteins could be detected in the *K. lactis* genome, they were to be cloned and characterized in comparative studies, using the approaches of reverse genetics, protein design, and cross-species complementation analyses.

2 Material and methods

2.1 Material

2.1.1 Chemicals and materials

Chemical compounds and other materials used in this study were purchased from the following companies: AppliChem (Darmstadt, Germany), BD (Franklin Lakes, USA), Boehringer Ingelheim (Ingelheim, Germany), Difco (Heidelberg, Germany), Eppendorf (Hamburg, Germany), Greiner BioOne (Frickenhausen, Germany), Omnilab (Bremen, Germany), Merck (Darmstadt, Germany), Roth (Karlsruhe, Germany), Serva (Heidelberg, Germany) and Sigma-Aldrich (St. Louis, USA).

2.1.2 Enzymes

Restriction enzymes were purchased either from Fermentas (St. Leon-Rot, Germany), New England Biolabs (Ipswich, USA) or Stratagene (Amsterdam, Netherlands). For cloning T4 DNA ligase and alkaline phosphatase were obtained from New England Biolabs. Amplifications of PCR products were performed using either “DreamTaqTM DNA Polymerase” or the “High Fidelity PCR Enzyme Mix” from Fermentas. For digestion of ascus walls, Zymolyase 20 T (MP Biomedicals, Solon, USA) was used.

2.1.3 Strains

2.1.3.1 Yeast strains

Tab. 2.1: *S. cerevisiae* strains employed and constructed in this work

Name	Genotype	Source
AS32	as DHD5 except *Scset4::KanMX*	Andrea Straede
AS93	as HAS100L7 except *Scset4::KanMX*	Andrea Straede
AS97	as HAS100L7 except *Scset4::KanMX*	Andrea Straede
DAJ24	as DHD5 except *Scinn1::SpHIS5/INN1*	(Jendretzki et al., 2009)
DAJ34	as DHD5 except *ScCYK3ΔSH3-3HA::SpHIS5/ScCYK3 ScHOF1ΔSH3-3HA::KanMX/ScHOF1*	(Jendretzki et al., 2009)
DHD5	*MAT a/α ura3-52/ura3-52 leu2-3,112/ leu2-3,112 his3-11,15/ his3-11,15*	(Arvanitidis and Heinisch, 1994)
HAJ37	as HD56-5A except *Scmyo1::SpLEU2*	(Jendretzki et

		al., 2009)
HAS100L7	pHPS100L integrated	Andrea Straede
HD56-5A	*MATα ura3-52 leu2-3,112 his3-11,15*	(Arvanitidis and Heinisch, 1994)
HNH1	as DHD5 except *Scnta1::KanMX/ScNTA1*	this work
HNH3	as DHD5 except *Scfig4::KanMX/ScFIG4*	this work
HNH11	as DHD5 except *Scfig4::KanMX/ScFIG4 Scwsc1::SpHIS3/ScWSC1 Scmid2::KlLEU2/ScMID2*	this work
HNH18	as HAS100L7 except *Scnta1::KanMX*	this work
HNH22	as HD56-5A except *KanMX-ScGAL1p-ScFIG4*	this work
HNH42	as HNH3 except *ScMID2-GFP-KanMX/ScMID2*	this work
HNH44	as HNH3 except *ScWSC1-GFP-KanMX/ScWSC1*	this work
HNH62	as DHD5 except *Scnta1::KanMX/ScNTA1 Scmpk1::SpHIS3/ScMPK1*	this work
HNH70	as DHD5 except *Scfig4::KanMX/ScFIG4 Scmpk1::SpHIS3/ScMPK1*	this work
HNH89	as DHD5 except *Scfig4::KanMXScFIG4 Scbck1::SpHIS3/ScBCK1*	this work
MALY4-3B	*Scbck1::SpHIS3/ScBCK1*	A. Lorberg
MALY5-2A	*Scmpk1::KlLEU2/ScMPK1*	A. Lorberg
PJ69-4A	*MATa trp1-901 leu2-3,112 ura3-52 his3-200 gal4Δ gal80Δ LYS::GAL1-HIS3 GAL2-ADE2 met2::GAL7-lacZ*	(James et al., 1996)

Tab. 2.2: *K. lactis* strains employed and constructed in this work

Name	**Genotype**	**Source**
CBS2359	*MATa*	(Kooistra et al., 2004)
DAS01	as KHO70 except *Klmyo1::KanMX/KlMYO1*	S. Albermann
KHO60	*MATa/MATα ura3-58/ura3, leu2/leu2, HIS3/his3::loxP, ADE2/ade2::loxP*	J. J. Heinisch
KHO70	*MATa/MATα, ura3-58/ura3, leu2/leu2, ADE2/ade2::loxP, HIS3/his3::loxP, ku80::loxP*	J. J. Heinisch
KNH7	as KHO60 except *Klhof1::ScLEU2/Klhof1::ScLEU2*	this work
KNH8	as KHO60 except *Klcyk3::ScURA3/KlCYK3*	this work
KNH9	as KHO60 except *Klhof1::ScURA3/KlHOF1*	this work
KNH10	as KHO60 except *Klinn1::ScLEU2/KlINN1*	this work
KNH14	as KHO70 except *KlCYK3-GFP-KanMX/KlCYK3*	this work
KNH15	as KHO70 except *KlHOF1-GFP-KanMX/KlHOF1*	this work
KNH19	as KHO60 except *Klhof1::loxP/KlHOF1*	this work
KNH20	as KHO70 except *KlINN1-GFP-KanMX/KlINN1*	this work
KNH21	as KHO70 except *KlMYO1-GFP-KanMX/KlMYO1*	this work

2.1.3.2 *Escherichia coli* strains

E. coli strain DH5α from Stratagene (*F´glnV44 thiA-1 Δ(argF-lac) U169 deoR endA1 gyrA96 hsdR17 recA1 supE44 (Φ80lacZΔM15) Nalr)* was used for cloning and strain BL21-DE3 (*fhuA2 [lon] ompT gal (λ DE3) [dcm] ΔhsdS λ DE3 = λ sBamHIo ΔecoRI-B int::(lacI::PlacUV5::T7 gene1) i21 Δnin5)* was used for heterologous expression of plasmids in *E. coli.*

2.1.4 Media and growth conditions

2.1.4.1 Media and growth conditions for *S. cerevisiae* and *K. lactis*

Rich medium (YEP):
1% (w/v) yeast extract
2% (w/v) peptone
Alternative carbon sources added:
2% (w/v) glucose, 2% (w/v) galactose or 2% (w/v) lactose
Whenever required, 1M sorbitol was added for osmotic stabilization.

Selective medium (SM):
0.67% (w/v) Yeast Nitrogen Base w/o amino acids (YNB)
0.06% (w/v) "complete supplement mixture" (CSM) w/o histidine, leucine, tryptophan, uracil and adenine
supplemented with the necessary amino acids and nucleotide bases according to (Zimmermann, 1975)
Alternative carbon sources added:
2% (w/v) glucose, 2% (w/v) galactose or 2% (w/v) lactose

For synthetic complete media (SC) all amino acids and nucleotide bases were added.

Sporulation plates:
1% (w/v) potassium acetate
3% (w/v) agar

Malt agar:
5% (w/v) malt extract
3% (w/v) agar

Plates for other experiments were prepared by adding 1.5% (w/v) agar to the media. Selection of *KanMX*-expressing cells was performed on YEPD plates containing 200 mg/l G418 for *S. cerevisiae* or 100 mg/l for *K. lactis*. Yeast cultures were incubated at 30 ℃, if not indicated otherwise.

Yeast cells were stored at 4 ℃ for short-term periods. For permanent storage, 500 µl of a fresh over night culture were mixed with 1 ml of sterile 33% (v/v) glycerol and frozen at -80 ℃.

2.1.4.2 Media and growth conditions for *Escherichia coli*

Rich media (LB): 1% (w/v) tryptone
0.5% (w/v) yeast extract
0.5% (w/v) NaCl

For plasmid selection cells were incubated with ampicillin or kanamycin at final concentrations of 50 mg/l or 20 mg/l, respectively. 1.5% (w/v) agar was added for plates. Bacteria were grown at 37 ℃. For blue/white screening, 100 µl of an X-Gal solution (10 mg/ml X-Gal in dimethylformamide) were spread onto the plate prior to streaking out the cell suspension.

Bacterial cultures were stored at 4 ℃ for a maximum of two weeks.

2.1.5 Plasmids

Tab. 2.2: Plasmids constructed in this work

Name	Backbone	Insert	Construction
pNH1	pJJH447	*ScNTA1*	amplified from HD56-5A with 05.175 and 05.176 cloned with *Bam*HI and *Sal*I
pNH2	pJJH447	*ScSET4*	amplified from HD56-5A with 05.175 and 05.176 cloned with *Bam*HI and *Sal*I
pNH56	YIplac128	*KlHOF3* (1012-1324)	Insert amplified with 08.105 and 08.106, cut with *Nsi*I and *Xba*I, vector cut with *Pst*I and *Xba*I
pNH62	YEp352	*KlCYK3*	subcloned with *Bam*HI and *Hind*III from pJJH1017
pNH74	pUK21	*ScCYK3*	subcloned with *Eco*RI and *Sph*I from pAJ024
pNH75	pCXJ20	*ScHOF1*	subcloned with *Pst*I and *Sac*I from pAJ023
pNH76	pCXJ18	*ScINN1*	subcloned with *Bam*HI and *Hind*III from pAJ022
pNH86	pNH107	*ScCYK3*	subcloned with *Eco*RI and *Sph*I from pAJ024
pNH92	pCXJ24	*KlCYK3*	subcloned with *Bam*HI and *Hind*III from pJJH1017
pNH93	pCXJ22	*ScCYK3*	subcloned with *Eco*RI and *Sph*I from pNH74

pNH94	pCXJ20	*ScCYK3*	insert subcloned with *Pst*I and *Spe*I from pNH74 vector cut with *Pst*I and *Xba*I
pNH95	pNH107	*ScHOF1*	subcloned with *Sac*I and *Sph*I from pAJ023
pNH101	pUK1921	*KlHOF1*	amplified from KHO60 with 09.136 and 09.137 cloned with *Bam*HI and *Pst*I
pNH102	pUK1921	*KlCYK3*	amplified from KHO60 with 09.138 and 09.139 cloned with *Bam*HI and *Pst*I
pNH105	pUK1921	*KlINN1*	amplified from KHO60 with 09.134 and 09.135 cloned with *Eco*RI and *Bam*HI
pNH107	pUC21	*KlLEU2*	amplified from pUG73 with 08.350 and 08.351 cloned with BamHI and HindIII
pNH110	pCXJ22	*KlHOF1*	subcloned with *Bam*HI and *Hind*III from pJJH1016
pNH112	pGBD-C2	*KlINN1*	subcloned with *Eco*RI and *Bam*HI from pNH105
pNH115	pCXJ24	*KlHOF1*	subcloned with *Bam*HI and *Hind*III from pJJH1016
pNH118	pGAD-424A	*KlCYK3*	subcloned with *Bam*HI and *Pst*I from pNH102
pNH120	pCXs22	*ScHOF1*	subcloned with *Pst*I and *Sac*I from pAJ023
pNH122	pCXJ22	*KlCYK3*	subcloned with *Bam*HI and *Hind*III from pJJH1017
pNH123	pGAD-424A	*KlHOF1*	subcloned with *Bam*HI and *Hind*III from pNH101
pNH126	pGBD-C1	*KlINN1* (421-1167)	amplified from KHO60 with 09.135 and 09.396, cut with *Sal*I and *Bam*HI, vector cut with *Sal*I and *Bgl*II

Tab. 2.3: Plasmids and vectors used during this work

Name	Description	Source
pAJ001	pKT128-*mCherry*	A. Jendretzki
pAJ015	pGAD424C-*ScHOF1*	A. Jendretzki
pAJ017	pGAD424B-*ScCYK3*	A. Jendretzki
pAJ022	YEP352-*ScINN1*	A. Jendretzki
pAJ023	YEP352-*ScHOF1*	A. Jendretzki
pAJ024	YEP352-*ScCYK3*	A. Jendretzki
pAJ025	pGBD-C2-*ScINN1*	A. Jendretzki
pCXJ20	*E.coli/S.cerevisiae/K.lactis* shuttle vector with *bla* resistance marker, *LEU2* marker, *Klori* and *KlCEN2* sequence	(Chen, 1996)
pCXJ22	*E.coli/S.cerevisiae/K.lactis* shuttle vector with *bla* resistance marker, *URA3* marker, *Klori* and *ScARS/CEN* sequence	(Chen, 1996)
pCXJ24	*E.coli/S.cerevisiae/K.lactis* shuttle vector with *bla* resistance marker, *LEU2* marker, *Klori* and *ScARS/CEN* sequence	(Chen, 1996)
pCXs22	pCXJ22 derivative, *Sph*I site substituted by *Bcl*I site	(Heinisch et al., 2010a)
pFA6a-KanMX6	*E.coli* vector for amplification of *KanMX* deletion cassettes	(Longtine et al., 1998)
pFA6a-	*E.coli* vector for amplification of C-terminal *GFP-*	(Longtine et

GFP(S65T)-KanMX6	tagging cassettes with the *KanMX* marker	al., 1998)
pFA6a-KanMX6-PGAL1	*E.coli* vector for amplification of the *GAL1* promoter preceded by the *KanMX* marker	(Longtine et al., 1998)
pGAD-424A	Yeast two-hybrid cloning vector for Gal4 activation domain fusions with *LEU2* and *bla* resistance marker	(James et al., 1996)
pGBD-C2	Yeast two-hybrid cloning vector for Gal4 DNA-binding domain fusions with *TRP1* and *bla* resistance marker	(James et al., 1996)
pJJH447	YEp352 derivative, MCS downstream of the *GAL1*-promoter	J. J. Heinisch
pJJH955L	pUG6 derivative for amplification of *ScLEU2* deletion cassette with flanking *loxP* sites	(Heinisch et al., 2010a)
pJJH955U	pUG6 derivative for amplification of *ScURA3* deletion cassette with flanking *loxP* sites	(Heinisch et al., 2010a)
pJJH959r	pCXJ24-*Cre*, encoding the bacteriophage P1 Cre-recombinase	(Heinisch et al., 2010a)
pJJH1008	YEp352-*KlINN1*	J. J. Heinisch
pJJH1009	YCplac33-*KlINN1*	J. J. Heinisch
pJJH1013	YCplac33-*ScPFK2p-KlINN1*	J. J. Heinisch
pJJH1016	YEp352-*KlHOF1*	J. J. Heinisch
pJJH1017	YEp352-*KlCYK3*	J. J. Heinisch
pJJH1204	YEp352-*KlINN1*$_{1-138}$-*ScINN1*$_{142-410}$	J. J. Heinisch
pJJH1205	YEp352-*ScINN1*$_{1-140}$-*KlINN1*$_{141-389}$	J. J. Heinisch
pSA3	YCpLac33-*KlMYO1*	S. Albermann
pSA12	pCXs22-*KlMYO1*	S. Albermann
pSA14	pCXs22-*KlMYO1-mCherry-KanMX*	S. Albermann
pSA15	pCXs22-*ScMYO1*	S. Albermann
pTD1	Yeast two-hybrid positive control plasmid, coding for a fusion of the SV40 large T-antigen (aa 86–708) and the Gal4 activation domain (aa 768–881) with *LEU2* and *bla* resistance marker	(Li and Fields, 1993)
pUC21	*E.coli* vector with *bla* resistance marker and *lacZ'* for blue/white screening	(Vieira and Messing, 1991)
pUG6	*E.coli* vector for amplification of *KanMX* deletion cassette and with *bla* resistance marker	(Gueldener et al., 1996)
pUG73	*E. coli* vector for amplification of *KlLEU2* deletion cassettes and with *bla* resistance marker	(Gueldener et al., 2002)
pUK1921	*E.coli* vector with *Kan*^{r} resistance marker and *lacZ'* for blue/white screening	(Vieira and Messing, 1991)
pUK21	*E.coli* vector with *Kan*^{r} resistance marker and *lacZ'* for blue/white screening	(Vieira and Messing, 1991)
pVA3	Yeast two-hybrid positive control plasmid, coding for a fusion of the murine p53 protein (aa 72–390) and the Gal4 DNA-binding domain (aa 1–147) with *TRP1* and *bla* resistance marker	(Iwabuchi et al., 1993)
YCpLac33	*S. cerevisiae- E. coli* shuttle vector with *ARS1/CEN4*	(Hill et al.,

	sequence, *URA3* and *bla* resistance marker and *lacZ'* for blue/white screenings	1986)
YEp352	*2 µm S. cerevisiae- E. coli* shuttle vector with *URA3* and *bla* resistance marker and *lacZ'* for blue/white screenings	(Hill et al., 1986)

2.1.6 Oligonucleotides

Oligonucleotides were purchased from Metabion international AG (Martinsried, Germany).

Tab. 2.4: Oligonucleotides employed in this work

No.	Name	5'-3' Sequence
98.125	BCK1-5	GCAAGCGGAGGTGGGTGAC
98.126	BCK1-3	GCCCTTCAGCGAAGAGG
03.17	YJL105Wendsph	GTACAGGAAGCAACCAACTAGG
03.18	YJL105start(Bgl)	CCGCAGATCTATGACTTCACCGGAATCACTATC
03.33	set4 "vor"	CAGGCACGAAGGCACACTCG
03.34	325vor	GCGTTAGTCATACAGTCCTG
03.35	325hinter	GGAGAACCGGGACCTCTAGC
03.44	KanMX-3´out	GTATTGATGTTGGACGAGTCGG
03.45	KanMX-5´out	GGAATTTAATCGCGGCCTCG
03.56	Set4 del5	GGCGAGAGCCGACATACGAG
03.57	Set4 del 3'2	GGTGCGTATATCGAGATGGC
03.68	YJL105 5'	CTTCACCGGAATCACTATCTTCTCGTCATATCAGGCAAG GAAGGACATACCTTCGTACGCTGCAGGTCGAC
03.69	YJL105 3'	CTTACAGTTGTGCATTATTGTATTAAAGTAAGATTTATTAG ATAGGGATTCCGCATAGGCCACTAGTGGATCTG
05.135	Mat1	AGTCACATCAAGATCGTTTATGG
05.136	Mat2	GCACGGAATATGGGACTACTTCG
05.137	Mat3	ACTCCACTTCAAGTAAGAGTTTG
05.172	NTA1del5	GCTAATAGACGCAATTCATGGTGCTAAGATGAGCACAAA ACTTTTAGTATCTTCGTACGCTGCAGGTCGAC
05.173	NTA1del3	CACCTAAACACTTCAAATTGGACCTCACGCAATATAGCG CCTTCTAGACCGCATAGGCCACTAGTGGATCT
05.174	NTA1vor	CACACCTCATTTGCCACATCCACC
05.175	NTA1nach	CATAATCTCCTAAATGACCCTC
06.15	SET4vorSal	GGCGGTCGACATGACTTCACCGGAATCACTATCTTCTCG
06.54	KlSLA2	GTATTCAACAACAGGAAATGGAAAAGG
06.55	KlMATa1	GGAGTCATGTGCGACAATGATATGGCAG
06.56	KlMATalpha1	GTAGATAAACAGACAAGAAAGAATTGGG
06.16	SET4nachBgl	GGCGAGATCTCAACTAATGATTGATGCTTAATCGTGG
06.17	Set4intern	GGTTCAAGTGACTCCAAGGATGAG
07.65	FIG4vorP	GTCAATGAATAGTTGTTAGACG
07.66	FIG5raus	GTTAATTCTCCACGGGGTACAGTAAG
07.73	FIG4F4	ATTGCATTAAACATATATATATAATATATACATGTATATAT TTCTTGTAAAGAATTCGAGCTCGTTTAAAC
07.74	FIG4R2	GACCCAGATGTGGTAAGTATACCTCCACCAAGGGTATGC TCCATTGCATCATTGTTCATTTTGAGATCCGGGTTTT

07.361	KlINN1vBgl	CCGAGTCACTAGTAATCCACC
07.362	KlINN1nEco	CCATCTTGTCGTATCATTAATCC
08.32	NTA1-F2	GGGTCTAGAAGGCGCTATATTGCGTGAGGTCCAATTTGA AGTGTTTAGGCGGATCCCCGGGTTAATTAA
08.33	NTA1-R1	GCCTCACTTAAATTCGGCCCTTACGGCGACATTTCAATA ACATAAATATGAATTCGAGCTCGTTTAAAC
08.67	KlINN1dis nach	GTTGGTGATACTCTCTGTAGATGG
08.68	Klinn1del5	ATGAGCAATAACGTGATAACCGGAGCAAATGGTCGATTA GATGTTTATGTCTTCGTACGCTGCAGGTCGAC
08.69	Klinn1del3	TTATAATGGTGGTGGTCTCCTCCTTGTTGGAGAAACCGA GCCTGATCTTGCCGCATAGGCCACTAGTGGATCTG
08.76	KlHOF1vorBam	GCGCGGATCCTTGCTGTTGAGCTGGTGCCTTGG
08.77	KlHOF1nachHind	GGCGAAGCTTCAATTTGCTAATAGTTTGAT
08.79	KlCYK3nachBam	CCGCGGATCCTCGCATTTATCTCTGAAATTAGTCC
08.83	KlINN1-R2	GGCTTCGAGAAGATAAGAGGAACATCTAATATGAGTAGT GATAGAATTCGAGCTCGTTTAAAC
08.85	KlCYK3-R2	GGTCTATATTCGAGATTAGTTATGACTGTGTCCGTCGATT GCAGAACACAGAATTCGAGCTCGTTTAAAC
08.87	KlHOF1-R2	GCTCAGCTTCAATTTGCTAATAGTTTGATGTCTATTGGAA GAGCAGAATTCGAGCTCGTTTAAAC
08.107	KlHOF1del5	ATGACTCAAATATACAACTATCAAGAAAGTTTCTGGGACG AAGACTTCGTACGCTGCAGGTCGAC
08.108	KlHOF1del3	TTACTTTCACGTAATTCATTGGTACCAGACCATGCTGTTT GTTACCGCATAGGCCACTAGTGGATCTG
08.109	KlCYK3del5	AAGACTAATTTCGCCTGGTCTGGTGAAAAGAAGGATGAT TTAGGTTTCTTCGTACGCTGCAGGTCGAC
08.110	KlCYK3del3	AATTCATTATCTACTCTCCCATTGCGCAAATTCACACCAT CCAGATCCGCATAGGCCACTAGTGGATCTG
08.175	KlINN1-F2	CGCCTTCTTCAAGATCAGGCTCGGTTTCTCCAACAAGGA GGAGACCACCACCATTACGGATCCCCGGGTTAATTAA
08.176	KlCYK3-F2	CTCCGATGCTGGATCTGGATGGTGTGAATTTGCGCAATG GGAGAGTAGACGGATCCCCGGGTTAATTAA
08.177	KlHOF1-F4	GGTAACAAACAGCATGGTCTGGTACCAATGAATTACGTG AAAGTAATAAATCGGATCCCCGGGTTAATTAA
08.197	GALpKlINN1-F4	CGAGGTGAATAAAAATACTTAACTCGACACTTAGTTAAAT AGCATAGCATGAATTCGAGCTCGTTTAAAC
08.198	GALpKlINN1-R2	CTCACATAAACATCTAATCGACCATTTGCTCCGGTTATCA CGTTATTGCTCATTTTGAGATCCGGGTTTT
08.199	KlMYO1-5`	GGCGGCATGCAATCACATGCTTCGGAAGCGG
08.200	KlMYO1-3`	GGCGCTCGAGTTGACGAAGGAAGAAGTGTCAAG
08.276	KlHOF1-vorGFP	CGTGTGACTATTGATGGTG
08.277	KlHOF1-nachGFP	GCAATCCTTCCTATCATTTAAAGTACAGATTTATCTGCTC AGCTTCAATTTGC
08.278	KlCYK3 vorGFP	CCATGGACCGGAATTTGAGTCG
08.279	KlCYK3 nachGFP	CCAAGGATATGAACTCATTCG
08.358	pCXJdelSphfor	GATTCGCACGCTGCAACGGAATGCATCACTAATGAAAAGC
08.359	pCXJdelSphrev	GCTTTTCATTAGTGATGCATTCCGTTGCAGCGTGCGAATC
08.371	Klmyo1del5	TTGACCAAATTACAGGTAAACTCAATTTGGGTACGAGGC TGTTAGGAAAACTTCGTACGCTGCAGGTCGAC
08.372	Klmyo1del3	AATGCTGTGCACACGAAATCAATGAAAAACTATGTTTCTA TAACATTTAACCGCATAGGCCACTAGTGGATCTG
08.373	KlMYO1-F5	GAAAGAAGACAATCCTTAGCTCAATCTACAGATGGTGTC

		TTCATTGGTGACGGTGCTGGTTTA
08.374	KlMYO1-R1	GCATTCATTAATTATTATTATAACATCAAATAAAGATATGT AAATAGCTAGAATTCGAGCTCGTTTAAAC
08.399	KlMYO1F2	CTTTCTGAAAGAAGACAATCCTTAGCTCAATCTACAGATG GTGTCTTCATTCGGATCCCCGGGTTAATTAA
09.29	KlMYO1-R1-mCherry	GCATTCATTAATTATTATTATAACATCAAATAAAGATATGT AAATAGCTACCGCATAGGCCACTAGTGGATCTG
09.134	KlINN1th EcoRIvor	GCGGCGGAATTCCGATTAAGTGTAATGAGC
09.135	KlINN1th BamHInach	CGGGATCCCGATTCCAAGGCTTCGAGAAG
09.136	KlHOF1 th BamHIvor	CGGATCCGCTATCGATTGAATGACTC
09.137	KlHOF1 th PstInach	CCGCCTGCAGGGAAAGCGTCCAAGGTGAAAG
09.138	KlCYK3 th BamHIvor	GCCGGATCCTTATGTCTCAGTCTGGACCAG
09.139	KlCYK3 th PstInach	GACCGCTGCAGGAGAATTCATTATCTACTCTCCC
09.399	ScINN1 Salforw	TTTAGTCGACGACGATCTTAACAAAGAAATGG

2.2 Methods

2.2.1 Transformation of yeasts and E. coli

2.2.1.1 Transformation of yeast cells according to Klebe (Klebe et al., 1983)

50 ml of a logarithmically growing cultures were pelleted at 3000 rpm for 3 min in a table-top centrifuge (Haraeus, Osterode, Germany), resuspended in 10 ml solution A (1 M sorbitol, 10 mM bicine, 3% ethylene glycol, pH 8.35) pelleted again, and resuspended in 3 ml solution A to be aliquoted into 200 µl portions in pre-cooled Eppendorf cups. Cups were then stored at -80 ℃ unt il use.

For transformation, 5 µl carrier DNA were added to frozen freeze-competent cells, in combination with 5 µl RFII (see transformation of *E. coli* for the recipe of the solution) and 5-15 µl sample-DNA. Cells were incubated at 37 ℃ for 5 min with frequent mixing. After adding 1 ml of solution B (40% PEG 4000, 200 mM bicine, pH 8.35) and inverting the reaction cup, it was incubated for 2 h at 30 ℃. Cells were collected by gentle centrifugation (5 min at 3500 rpm), the supernatant was discarded and cells were resuspended in the remaining droplet and streaked onto selective plates. For geneticin-selection, cells were resuspended in 4 ml YEPD, incubated over night at

room temperature, harvested and spread on G418-plates. Plates were incubated for 3-4 days at 30 ℃.

2.2.1.2 Transformation of yeasts according to Gietz (Gietz et al., 1995)

50 ml YEPD were inoculated to an OD of 0.2 and grown until an OD of 1.2-1.5 was reached. The culture was centrifuged for 3 min at 3000 rpm at room temperature. After discarding the supernatant, the pellet was washed with 25 ml sterile water and cells were collected again. The supernatant was discarded, the pellet resuspended with 1 ml 100 mM LiAc, centrifuged and resuspended in 400 µl 100 mM LiAc. The cell suspension was aliquoted into 50 µl portions, which were centrifuged for 3 min at 3000 rpm. After removing the supernatant thoroughly using a Gilson pipette, the pellet was carefully overlaid with 50% PEG 3350. Onto this layer, 36 µl LiAc, 5 µl carrier DNA and 5-15 µl DNA were pipetted. After resuspension of the cells, the mixture was incubated for 30 min at 30 ℃ and for a dditional 20 min at 42 ℃. Cells were pelleted, washed with sterile water and streaked onto selective plates. For geneticin-selection, cells were incubated in 5 ml YEPD over night at room temperature without agitation and streaked onto G418-YEPD plates.

2.2.1.3 *E. coli* transformation (Hanahan, 1983)

50 ml LB-medium were inoculated with a fresh *E. coli* over night culture to an OD of 0.05 and incubated with shaking at 37 ℃ to reach a n OD of 0.4 – 0.6. Cells were kept on ice for 1-2 hours, collected by centrifugation for 10 min at 3000 rpm, washed with 20 ml of pre-cooled RFI-solution (100 mM RbCl, 50 mM $MnCl_2$, 30 mM KAc, 10 mM $CaCl_2$, 15% (w/v) glycerol, pH 5.8) and again incubated on ice for 1-2 hours. Cells were harvested and resuspended in 4 ml pre-cooled RFII-solution (10 mM RbCl, 10 mM MOPS, 75 mM $CaCl_2$, 15% glycerol, pH 6.8). After incubation for 15 min on ice, cells were aliquoted in 100 µl portions and frozen at –70 ℃.

For transformation, 100 µl *E. coli* cells were thawed on ice and either 1 µl of plasmid DNA or a complete ligation batch (in a total volume of 20 µl) was added. Cells were incubated on ice for 20 min, followed by a heat shock for 1 min at 42 ℃. After adding 1 ml of LB-medium, cells were allowed to regenerate for 1 hour at 37 ℃, pelleted and streaked onto a LB-plate containing a suitable antibiotic.

2.2.2 Sporulation and tetrad analysis

In order to induce sporulation of diploid *S. cerevisiae* strains, 3-4 ml of stationary cells were harvested by centrifugation for 3 min at 3,000 rpm. The supernatant was discarded and cells were resuspended in the remaining medium, dropped onto potassium acetate plates, and incubated for 2-4 days at 30 ℃. After microscopical confirmation of tetrad formation, a toothpick was used to resuspend them in 0.14 mg/ml Zymolyase solution (7 µl of 10 mg/ml Zymolyase 20T in 500 µl distilled water). After 10 min of incubation at room temperature, 10-15 µl of cell suspension were spread in a line on one side of a YEPD plate and tetrads were dissected using the micromanipulator „MSM System“ by Singer Instruments (Watchet, UK). Marker segregation was tested after growth for 3-4 days at 30 ℃ and preparation of a master plate by replica-plating onto different appropriate drop-out media.

2.2.3 Mass spore analysis

For depletion of vegetative non-sporulated *K. lactis* cells, a sample of starved cells was taken from the potassium acetate plate, resuspended in 0.4 mg/ml Zymolyase solution (20 µl 10 mg/ml Zymolyase 20T in 500 µl distilled water) and incubated for 1 h at room temperature. After mixing thoroughly, serial dilution samples of the cell suspension (100 µl of 1:1, 1:10 and 1:100 dilutions) were spread onto SM-ura plates.

2.2.4 Generation of diploid *K. lactis* strains

Two logarithmic growing cultures of haploid yeast strains with opposite mating types were dropped onto a malt agar plate, partially mixed and stroke out for distribution of cells. After incubation for 1-2 days at 28 ℃ singl e colonies were patched and replica plated on selective plates to determine the genotype.

2.2.5 Analysis of DNA

2.2.5.1 Purification of plasmids from *E. coli*

Isolation of plasmid DNA from *E. coli* was performed by using the “High Pure Plasmid Isolation Kit” (Roche, Mannheim), following the instructions of the manufacturer.

2.2.5.2 Isolation of plasmids from *S. cerevisiae*

For preparation of plasmid DNA from *S. cerevisiae*, the “High Pure Plasmid Isolation Kit” (Roche, Mannheim) was used, except one additional step. After resuspension of the yeast cells in 250 µl suspension buffer, ~100 µg of glass beads were added and cells were broken by shaking on an “IKA-Vibrax-VXR” (IKA, Staufen, Germany) at 4 ℃ for 10-20 min. The remaining steps were perfor med following the manufacturers instructions. After elution of the plasmid DNA from the columns with 50 µl buffer, 20 µl were used to transform *E. coli* for plasmid amplification and further analyses.

2.2.5.3 Restriction, ligation and dephosphorylation of DNA

Enzymes for restriction, ligation and dephosphorylation were applied according to the instructions provided by the manufacturers.

2.2.5.4 Separation of DNA fragments by agarose gel electrophoresis

For separation and analysis of DNA fragments, agarose gel electrophoresis was performed. Depending on the size of DNA fragments, a 0.8-2.0% (w/v) agarose gel was chosen, dissolving agarose in 1x TAE buffer (40 mM Tris-HCl pH 8.3, 20 mM acetic acid, 1 mM EDTA). Electrophoresis was performed using the “Power Pack P25” from Biometra (Göttingen, Germany), applying 100 V for 45-60 min. For comparison of DNA sizes, different markers were employed; either the “2log DNA ladder”, the “100 bp ladder” (New England Biolabs, Ipswich, USA), or a standard prepared from DNA of bacteriophage λ, digested with the restriction endonucleases *Eco*RI/*Hind*III. Gels were incubated in 0.5 µg/ml ethidium bromide solution for 15 min and DNA was visualized by exposure to UV-light (λ = 366 nm) and digitally documented on a “GelDoc-It Ultraviolet Transilluminator” (UVP, Upland, USA).

2.2.5.5 Isolation of DNA fragments from agarose gels

DNA fragments or PCR products were purified using the “High Pure PCR Product Purification Kit” (Roche, Mannheim, Germany).

2.2.5.6 Preparation of genomic DNA from yeast

A tooth pick of yeast cells from a stationary colony was thoroughly suspended in 100 µl extraction buffer (50 mM NaCl, 1 mM EDTA pH 8, 10 mM Tris-HCl pH 8, 0.5% Triton X-100) and mixed with 100 µl phenol/chloroform/isoamyl alcohol (ratio 24:25:1). From this point on, samples were kept on ice and further steps only took place in pre-cooled instruments or at 4 °C. About 1 00 µl of glass beads were added, samples were shaken for 10 min on a “Vibrax VXR basic” (IKA, Staufen, Germany) and centrifuged for 15 min at 13000 rpm. 10 µl of the upper aqueous phase were mixed with 90 µl distilled water and stored at -20 °C. For usage as template for PCR 5 µl were employed.

2.2.5.7 Polymerase Chain Reaction (PCR)

In order to amplify DNA fragments, PCRs were performed with either “DreamTaqTM DNA Polymerase” (for confirmatory assays) or “High Fidelity PCR Enzyme Mix” (Fermentas, St. Leon-Rot, Germany; for subcloning procedures), according to the manufacturers instructions. A standard amplification program consisted of a first denaturation step (2 min at 95 °C), 33 cycles of de naturation (30 s at 95 °C), annealing (30 s at 58 °C) and elongation (1 min/kb desired product at 72 °C for up to 3 kb or at 68 °C for longer fragments) and a termin al elongation step at 72 °C for 10 min. All PCRs were done in a “Personal Cycler” from Biometra (Göttingen, Germany).

2.2.5.8 Sequencing

DNA sequences were obtained from Scientific Research and Development GmbH (SRD; Bad Homburg, Germany). Plasmid DNA was purified with the “High Pure Plasmid Isolation Kit” (Roche, Mannheim, Germany), diluted to 150-300 ng/µl, and 10 pmol/ml of the respective oligonucleotides in a final volume of 7 µl.

2.2.6 Analysis of proteins

2.2.6.1 Crude extracts

25 ml of a logarithmically growing culture were centrifuged for 3 min at 3000 rpm, washed twice with 5 ml water and resuspended in 500 µl potassium phosphate buffer (50 mM potassium phosphate, pH 7.0). After centrifugation and resuspension in 500 µl of the same buffer, 200 µg of glass beads were added and cells were broken by shaking on “Vibrax-VXR basic” (IKA, Staufen, Germany) at 4 °C for 20-30 min. After transfer of the supernatant to a new cup, the glass beads were rinsed with 500 µl KP_i-buffer, which were added to the previous supernatant. The suspension was centrifuged for 10 min at 4 °C at 13,000 rpm and the clarified supernatant was transferred to a new cup. Whole cell extracts were kept at 4 °C for a maximum of 3 h before use.

2.2.6.2 β-galactosidase activity assay

For determination of the specific β-galactosidase activity, 950 µl LacZ-buffer (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 1 mM $MgSO_4$, 2 mg/ml ONPG, pH 7.0) were pre-heated to 30 °C and mixed with 50 µl of crude extract. The colourless solution was incubated at 30 °C, until it turned yellow. To stop the reaction, 500 µl of 1 M Na_2CO_3 were added, and the absorption was measured against a reference blank at 420 nm. Specific β-galactosidase activities were calculated according to the following equation:

$$[\text{mU/mg}] = (\Delta E/\text{min} * V_{total} * 10^6)/(\varepsilon * m_{protein} * V_{CE} * d)$$

ΔE_{420nm} = extinction at 420 nm
ε = extinction coefficient = $4.5*10^3$ mol^{-1} cm^{-1}
V_{total} = total volume = 1500 µl
V_{CE} = volume of crude extract = 50 µl
$m_{protein}$ = protein concentration [mg/ml]
d = thickness of the cuvette = 1 cm

2.2.6.3 Measurement of protein concentration

Determination of protein concentration in crude extracts was performed by the "Micro-Biuret" method as described in (Itzhaki and Gill, 1964).

2.2.7 Cre-recombinase mediated recombination

For induction of recombination between *loxP*-sites, plasmid pJJH959r (Heinisch et al., 2010a) was introduced into the respective *K. lactis* strain. This plasmid encodes the Cre-recombinase under the control of the *GAL1*-promoter on a *K. lactis* vector with a *ScLEU2* selection marker. Transformants were selected for leucine prototrophy and inoculated in 3 ml YEPLac in order to induce expression of the Cre-recombinase gene. Cultures were re-inoculated each morning for 3 days in fresh YEPLac media to an OD_{600} of 0.1. Serial dilutions were then plated onto YEPD to obtain single colonies, which were subjected to replica plating and PCR analyses to ensure that the desired recombination event occurred at the chromosomal locus and that the plasmid carrying the Cre-recombinase genes had been cured.

2.2.8 Drop dilution assays

To examine phenotypes of yeast strains in serial drop dilution assays, cells were inoculated in liquid media and incubated over night at permissive temperatures. Cultures were diluted to an OD_{600} of 0.15 in fresh media, incubated for another 5-6 h and adjusted to an OD_{600} of 0.6. Serial dilutions from 10^{-1} - 10^{-4} were prepared and 3 µl of each were dropped onto the test media and incubated as indicated. Growth was documented after 2-5 days by scanning. To adjust brightness and contrast, the Corel Photo Paint program was employed.

2.2.9 Yeast two-hybrid analysis

For determination and comparison of protein-protein interactions the yeast two-hybrid system was employed. The coding sequences of proteins of interest were either fused to the DNA binding domain or activation domain of the *S. cerevisiae* transcription factor *GAL4.* Resultant plasmids were introduced into the strain PJ96-4A (James et al., 1996) and transformands were selected on plates lacking leucine and tryptophan. For first qualitative analyses, transformands were patched on a

master-plate (SM-Leu-Trp) and replica plated on plates additionally lacking adenine or histidine. The reporter genes *HIS3* and *ADE2* are only expressed when Gal4 is activated which in turn only occurs when the tested proteins are interacting.
In order to allow a more precise comparison, single drop assays (see 2.2.8, without serial dilutions) were performed or crude extracts were prepared for determining of specific β-galactosidase-activities (see 2.2.6.1 and 2.2.6.2, respectively).

2.2.10 Cell imaging and microscopy

2.2.10.1 Microscope settings

The “Axioplan2” microscope equipped with objectives “Plan-Apochromat 100x / 1.45 NA Oil DIC” (Carl Zeiss AG, Feldbach, Switzerland) and accordant filter sets from Chroma (Rockingham, USA) was used. As a light source for fluorescent microscopy the 100 W HBO lamp from OSRAM AG (Augsburg, Germany) was employed. The camera set-up consisted of the cooled charged-coupled device camera “CoolSNAP HQ” (Roper Scientific, Tucson, USA) and the shutter control system “MAC200” (LUDL, Hawthome, USA). For operating the microscope the software “MetaMorph v6.2” (Universal Imaging Corporation, Downington, USA) was employed.

2.2.10.2 Acquisition and processing of images

For microscopical examinations, cells were incubated in SM- or SC-medium for several hours at 30 °C. Images were taken as single planes using differential-interference-contrast (DIC) illumination for brightfield acquisition and fluorescence images were scaled by MetaMorphs “Scale Image” command.
For visualisation of GFP-signals excitation and emission were performed at ~480 nm and ~510 nm, respectively, whereas mCherry was excited at ~550 nm and emitted at ~580 nm. Depending on the signal strengths, excitations took place for 500-2000 ms.
For deconvolution the software program “Huygens Essential” (Scientific Volume Imaging B. V., Hilversum, Netherlands) was used.

2.2.10.3 Time-lapse analyses

In order to perform time-lapse microscopy, special slides were employed which contain a cavity filled with ~200 µl of time-lapse medium (25% SC medium, 10%

glucose, 0.5% agarose). Onto this matrix, 5 µl of cells were spread, covered with a cover slip and air-tight sealed with rubber cement. For incubation at constant temperatures the „Delta T4 Culture Dish Controler“ (Bioptechs, Beck Road, USA) was employed, requiring an altered set-up for the microscope slides. One long cover slip was evenly coated with ~500 µl time-lapse medium and 15 µl of cell culture were spread. With a second long cover slip and rubber cement the sample was thoroughly sealed, mounted onto the culture dish and connected to the temperature sensor. Interval settings were adjusted as indicated.

2.2.10.4 Chitin staining

For visualization of chitin containing compartments 50 µl of yeast cultures were incubated with Calcofluor White (50 µg/ml final concentration) for 3-5 min. To diminish the excess amount of chitin, cells were washed twice with water and afterwards examined under the microscope. Fluorescence was excited for ~20-50 ms at 370 nm and emitted light was detected at 440 nm.

3 Results

This work can be divided into two different projects of which one focuses on yeast cell wall integrity and the other on the regulation of cytokinesis: In the first part of the thesis, three putative negative regulators of CWI signaling isolated previously were analyzed in further detail. The second and main part of this thesis concentrates on the regulation of cytokinesis in the milk yeast *K. lactis*, which is closely related to the bakers yeast *S. cerevisiae*.

3.1 Further analysis of putative negative regulators of CWI signaling in *S. cerevisiae*

In a previous Ph.D. thesis, Hans-Peter Schmitz designed a genetic screen and employed a transposon mutagenesis to isolate putative negative regulators of CWI signaling in *S. cerevisiae*. Three of these genes, namely *NTA1, SET4* and *FIG4*, were here investigated in further detail to determine the CWI stress-related phenotypes of strains carrying either complete deletions or, *vice versa,* overexpressing the genes from the inducible *GAL1* promoter.

The original genetic screen yielding the transposon mutants was based on a *lacZ*-based reporter construct and the concomitant development of blue colored colonies on X-Gal indicator plates. In order to verify the preliminary data obtained on the originally isolated transposon-insertion mutants, haploid deletions lacking the entire ORFs of *NTA1, SET4* and *FIG4* were constructed by one-step gene replacement with PCR-generated marker cassettes as described in materials and methods (section 2.2.5.7). These mutants (*nta1, set4, fig4*) were first crossed to a strain carrying an integrated reporter construct (pHPS100L, strain HAS100L7) and suitable segregants (e.g. *nta1::KanMX 100L7*) were used to determine the specific β-galactosidase activities in crude extracts (Table 1). Only a strain carrying the *fig4* deletion displayed a clearly increased activity after heat stress as compared to the wild-type control. On the other hand, nta1 deletions seemed to increase the basal level of pathway activity and were not further induced by heat stress.

Tab. 3.1: The β-galactosidase activity in the *Scfig4Δ* LexA100L7 integrant is increased upon heat stress.

strain	30℃	37℃
HAS100L7	2.26 ± 0.80	4.81 ±0.98
HNH18 (*nta1Δ*)	8.03 ±0.85	9.95 ±1.05
AS97 (*set4Δ*)	1.96 ±2.10	2.55 ±1.26
AS93 (*fig4Δ*)	4.01 ±1.31	12.75 ±0.07

Strains producing the LexA-Rlm1 reporter construct and carrying a deletion of either ScNTA1, *ScSET4* or *ScFIG4* were incubated at 30℃ and 37℃ over night. Crude extracts were harvested and subjected to β-galactosidase-activity assays (see section 2.2.6.2). Mean values with standard deviation from three measurements are given in [mU/mg].

In a next step, the deletion mutants were assessed in drop dilution assays for their sensitivities to different cell wall stress conditions. As controls, either the wild-type or deletion strains defective in either of two kinases of the CWI signalling cascade (Bck1 and Mpk1) were employed. The deletion of a putative negative regulator of the CWI pathway would be expected to display an increased resistance to cell wall perturbing agents (e.g. caffeine or Congo red) as compared to the wild-type control, whereas both kinase mutants should be hyper-sensitive. The controls behaved as expected, whereas none of the three deletion mutants (*nta1, set4, fig4*) significantly differed in its growth behaviour as compared to the wild-type under the conditions tested (Fig. 3.1; Heinisch et al., 1999).

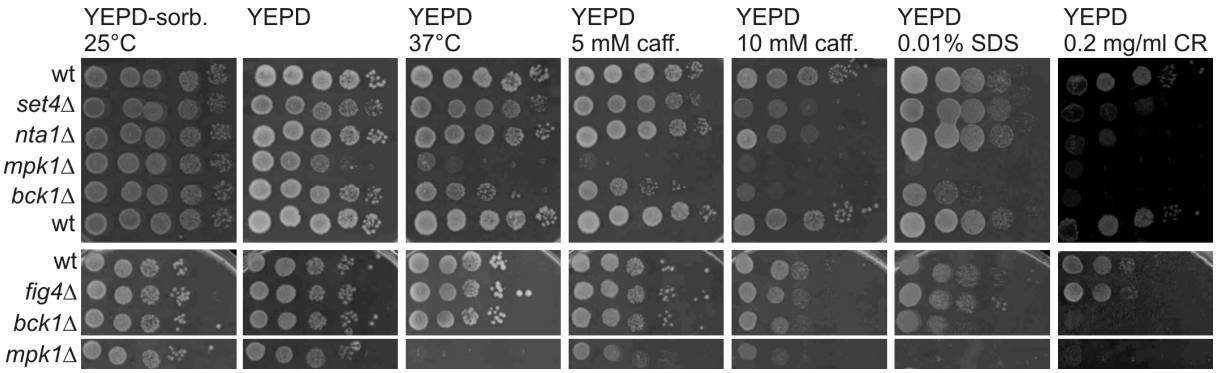


Fig. 3.1: Deletions of *ScSET4*, *ScNTA1* and *ScFIG4* do not affect resistance to cell wall perturbing agents. Segregants from strains carrying the deletion of either *ScSET4* (AS32), *ScNTA1* (HNH1) or *ScFIG4* (HNH3) were subjected to drop dilution assays as described in section 2.2.8. As controls the wild-type strain HD56-5A (wt) as well as *Scbck1*Δ (MALY4-3B) and *Scmpk1*Δ (MALY5-2A) strains were employed. Plates were incubated at 30℃ or as indicated and scanned after 2-3 days.

Two of the deletion mutants, *fig4* and *nta1*, where then used for epistasis analyses in conjunction with CWI pathway mutants. For this purpose, a strain carrying the *fig4* deletion was crossed to strains carrying either a *wsc1 mid2* double deletion, a *bck1* deletion, or a *mpk1* deletion (lacking the genes encoding the two main sensors of the CWI pathway, the MAPKKK and the MAPK, respectively). Segregants with the

desired double and triple deletions were obtained after sporulation and tetrad analysis and subjected to drop dilution assays on media containing different cell wall stress agents (Fig. 3.2). As expected, the *mpk1, bck1* and *wsc1 mid2* deletion strains are highly sensitive to cell wall stress. The additional deletion of *FIG4* restored viability to both a *mpk1* and a *wsc1 mid2* deletion strain, but not to the *bck1* deletion.

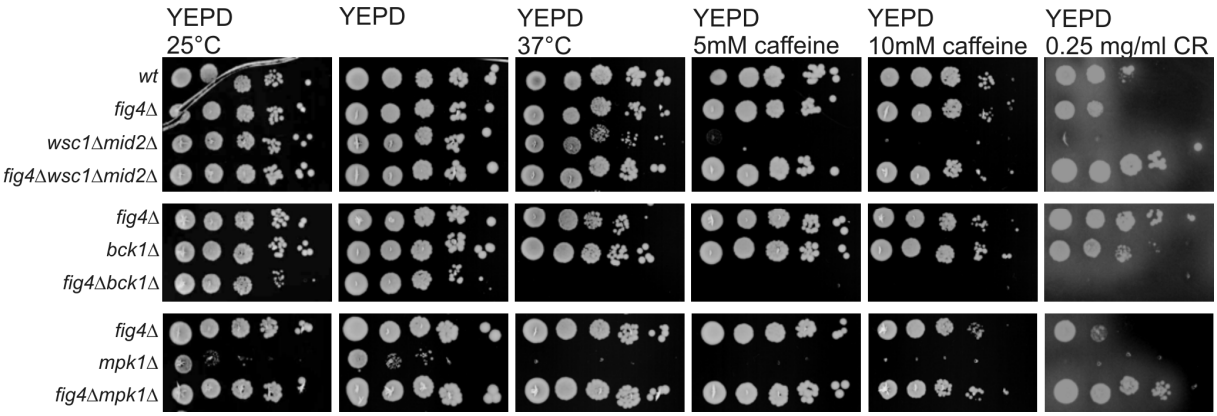


Fig. 3.2: Additional deletion of *ScFIG4* restores viability to *wsc1Δ mid2Δ* and *mpk1Δ* deletion mutants.
Segregants of strains carrying the deletion of *Scfig4* together with deletions of *wsc1Δ mid2Δ*, *bck1Δ* or *mpk1Δ* (strains HNH11, HNH89 and HNH70, respectively) were employed for serial drop dilution assays (see 2.2.8). Plates were incubated at 30℃ or as indicated and gr owth was documented after 3 days.

On the other hand, a *nta1 mpk1* double deletion did not differ in its growth behaviour from a single *mpk1* deletion (Fig. 3.3). It should be noted that drop dilution assays with CWI pathway mutants are frequently prone to significant variations, which are presumably caused by minute differences in medium compositions. Thus, although promising, both the suppression phenotype of the *fig4* deletion and the lack of synthetic phenotypes of the *nta1* deletion need to be verified in further analyses.

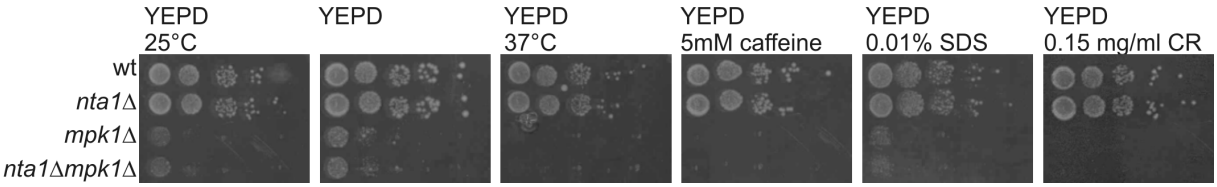


Fig. 3.3: Additional deletion of *ScNTA1* does not repress the phenotype of *Scmpk1* deletion mutants.
Segregants of sporulated strain HNH62 (*Scnta1::KanMX/ScNTA1, Scmpk1::SpHIS3/ScMPK1*) were subjected to serial drop dilution assays (see 2.2.8). Plates were incubated at 30℃ or as indicated and scanned afte r 3 days.

Whilst a deletion mutant in a putative negative regulator is expected to increase the resistance to cell wall stress, the overexpression of the respective gene should result in an increased sensitivity to such agents. To investigate if the overexpression of *NTA1, SET4* or *FIG4* is consistent with this hypothesis, the respective genes were expressed under the control of the inducible *GAL1p* promoter using multicopy plasmids. Plasmids carrying either *NTA1* or *SET4* under the control of the *GAL1*

promoter (pNH1 and pNH2) were introduced into the wild-type strain HD56-5A and transformants were again tested in serial drop dilution assays.

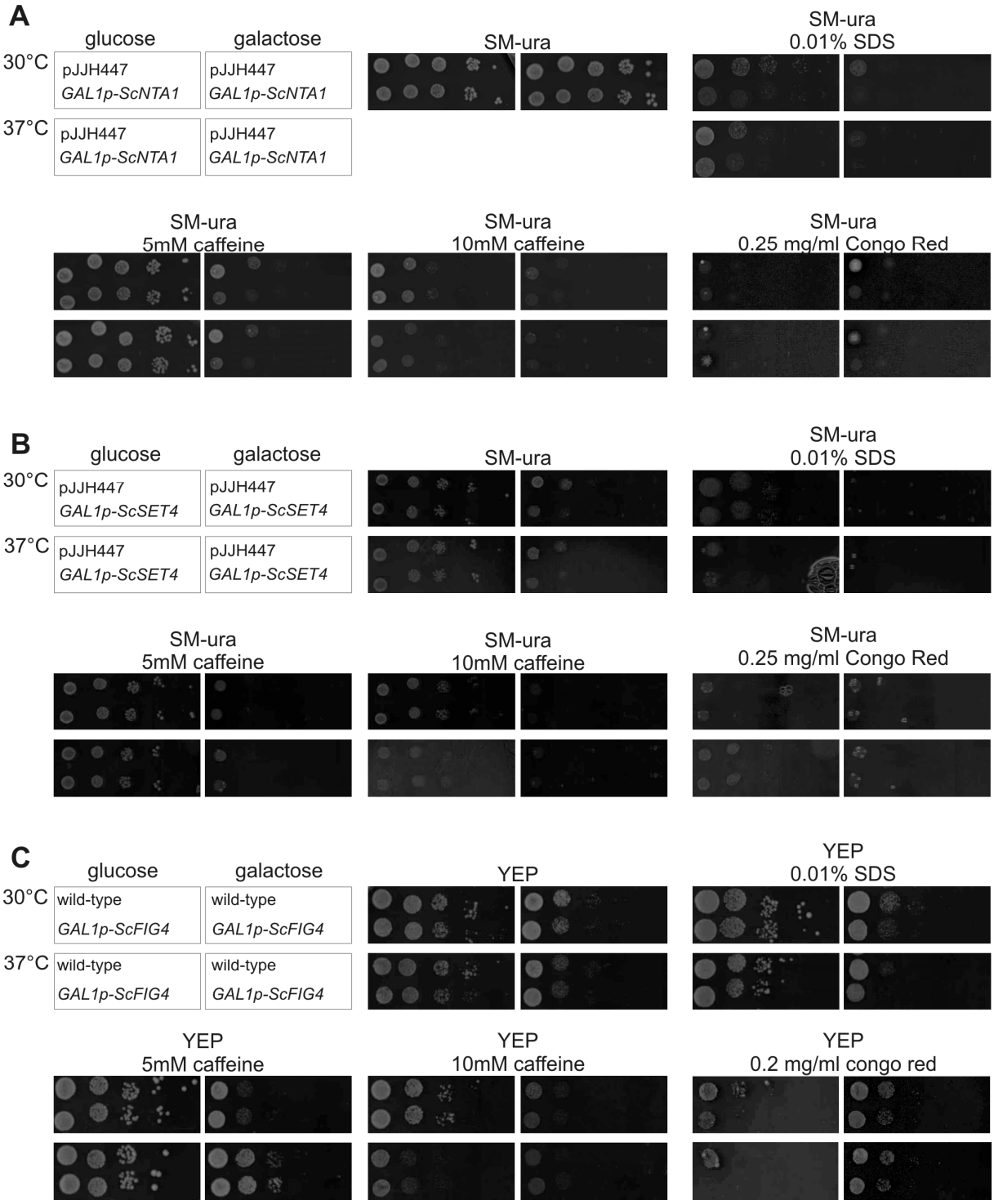


Fig. 3.4: Overexpression of neither *ScNTA1*, *ScSET4* nor *ScFIG4* displays an altered phenotype.
A + B: Wild-type strain HD56-5A was transformed with empty vector pJJH447 (upper row in each panel) or *GAL1p*-fusion plasmids (lower row in each panel; A: pNH1 [pJJH447-*ScNTA1*], B: pNH2 [pJJH447-*ScSET4*]). Transformants were subjected to serial drop dilution assays (see 2.2.8) on SM-uracil plates containing either glucose (left panels) or galactose (right panels) as carbon source. Plates were incubated for two (glucose) or three days (galactose) at the indicated temperatures. **C:** Wild-type strain HD56-5A (upper row) and strain HNH22 (*GAL1p-ScFIG4* fusion, lower row) were subjected to serial drop dilution assays (see 2.2.8) on rich media plates containing either glucose (left panels) or galactose (right panels) as carbon source. Plates were incubated for two (YEPGlc) or three days (YEPGal) at the indicated temperatures.

Strains carrying the original vector pJJH447 without any insertion were employed as controls. In the case of *FIG4*, a chromosomal *GAL1p* fusion was introduced into the wild-type strain HD56-5A by homologous recombination to yield strain HNH22. Phenotypes were assessed on plates containing either glucose (repressing conditions) or galactose (inducing conditions) with different stress agents incubated at either 30℃ or 37℃. As evident from Fig. 3.4, n one of the transformants differed in its growth behaviour from the strains with the vector control or the wild-type strain, respectively.

In further analyses for candidate interactions, the distribution of two GFP-tagged cell wall sensors (Wsc1-GFP or Mid2-GFP) did not differ in a *fig4* deletion as compared to *FIG4* wild-type cells. Moreover, the N-terminal amidase Nta1 did not interact with the GEF Rom1 (a putative target protein deduced from its amino acid sequence) in a yeast two-hybrid assay (data not shown).

Thus, albeit the data obtained so far indicate some indirect connections between the three genes investigated here and the CWI signaling pathway, the evidence was judged as too vague to invest more time and effort in this line of research.

3.2 Characterization of cytokinesis regulators in *Kluyveromyces lactis*

The next chapters will deal with the characterization of the genes *KlCYK3, KlHOF1, KlINN1* and *KlMYO1* in *K. lactis*, whose homologues of *S*. *cerevisiae* are being investigated in our laboratory in a different line of research.

3.2.1 Alignments and deletions

3.2.1.1 *Kl*Cyk3 is an essential protein

*Sc*Cyk3 is a known regulator of yeast cytokinesis. It contains a so-called SARC-homology (SH3) domain, which mediates interactions with proteins containing proline-rich motifs (PRMs). The gene which encodes its homologue in *K. lactis* is located on chromosome V and spans 1167 bp, with a deduced primary sequence of 388 amino acids. The overall identity between the *K. lactis* and *S. cerevisiae* homologues is 42%, being significantly higher when comparing only the SH3-domains (60%; Fig. 3.5)

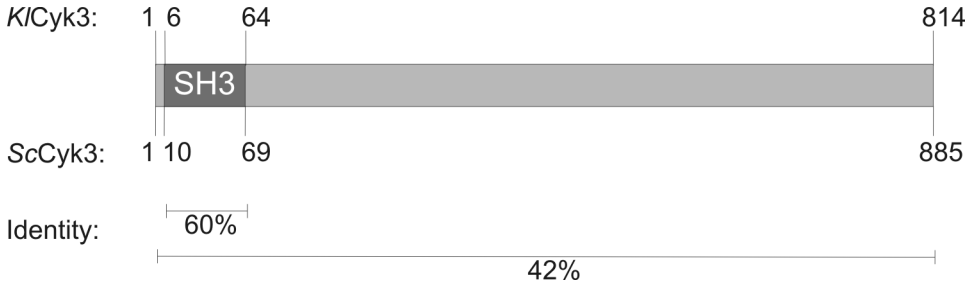


Fig. 3.5: *Kl*Cyk3 and *Sc*Cyk3 share 42% identity.
The Cyk3-homologues from *K. lactis* and *S. cerevisiae* share a similar modular arrangement with 42% overall identity and 60% within the SH3-domains. The schematic drawing indicates the respective spans by giving the first and last amino acids.

To gain further information about the *in vivo* function of *Kl*Cyk3, the heterozygous diploid deletion strain (KNH8, *Klcyk3::ScURA3/KlCYK3*) was constructed and subjected to tetrad analysis. From a total of nine tetrads separated, seven showed a 2:0 segregation for the ability to produce colonies, with none of the tetrads generating more than two colonies (Fig. 3.6). None of the viable segregants carried the *ScURA3* deletion marker used originally for the gene replacement. This clearly indicates that the two non-viable segregants harbour the *Klcyk3* deletion, which is obviously lethal, in contrast to its counterpart in *S. cerevisiae*.

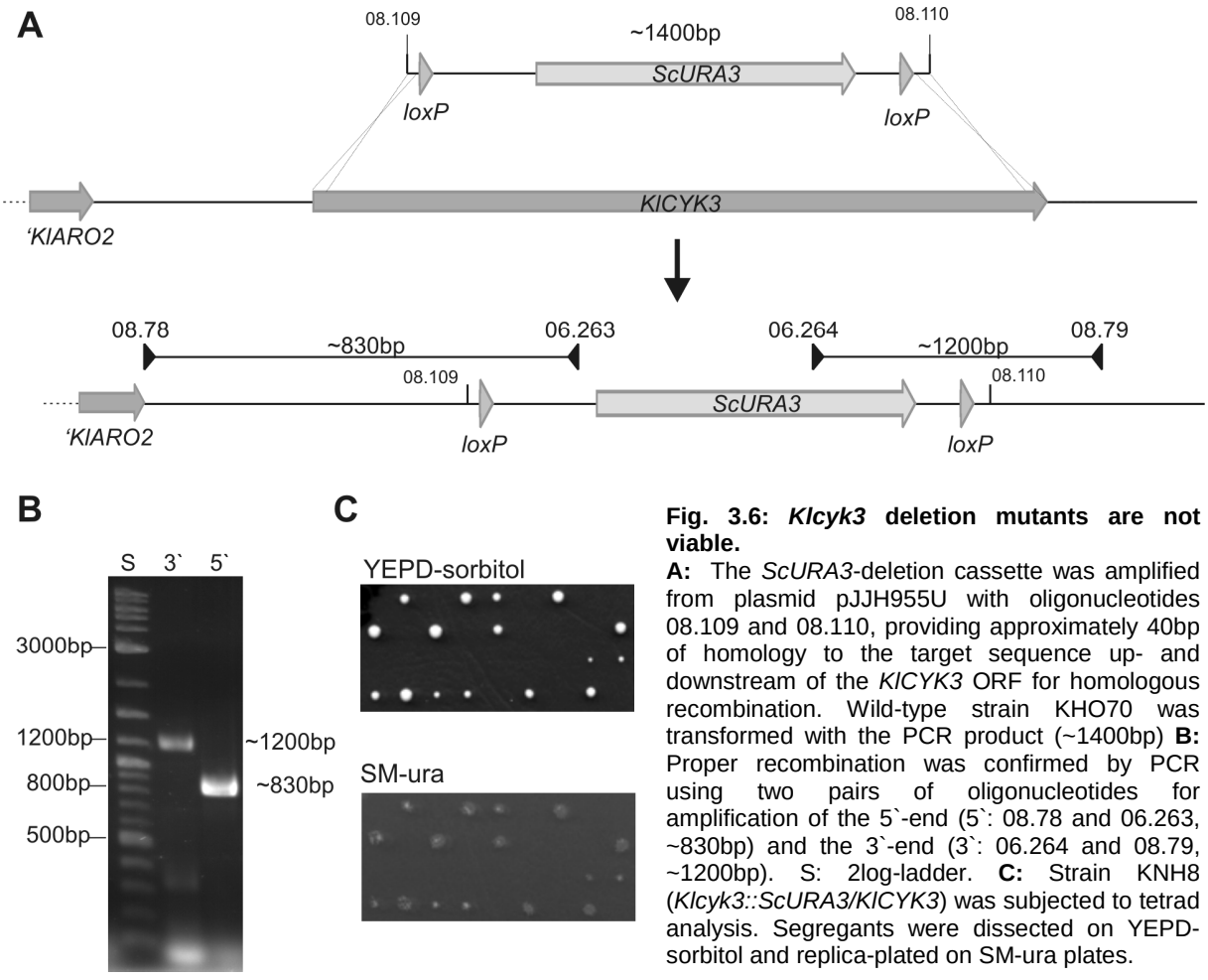


Fig. 3.6: *Klcyk3* deletion mutants are not viable.
A: The *ScURA3*-deletion cassette was amplified from plasmid pJJH955U with oligonucleotides 08.109 and 08.110, providing approximately 40bp of homology to the target sequence up- and downstream of the *KlCYK3* ORF for homologous recombination. Wild-type strain KHO70 was transformed with the PCR product (~1400bp) **B:** Proper recombination was confirmed by PCR using two pairs of oligonucleotides for amplification of the 5`-end (5`: 08.78 and 06.263, ~830bp) and the 3`-end (3`: 06.264 and 08.79, ~1200bp). S: 2log-ladder. **C:** Strain KNH8 (*Klcyk3::ScURA3/KlCYK3*) was subjected to tetrad analysis. Segregants were dissected on YEPD-sorbitol and replica-plated on SM-ura plates.

3.2.1.2 *Kl*Hof1 is an essential protein

In *S. cerevisiae*, another key regulator of cytokinesis is Hof1 (homolog of Cdc fifteen; originally identified in *Schizosaccharomyces pombe*; Marks et al., 1992). This protein is involved in the regulation of actomyosin ring constriction during cytokinesis, as well as in the proper localization of septins. It also interacts with formins, which in turn organize the actin cytoskeleton. *Sc*Hof1 contains a C-terminal SH3-domain and a FCH-domain (Fes/CIP4 [Cdc42 interacting protein 4] homology) at its N-terminus.

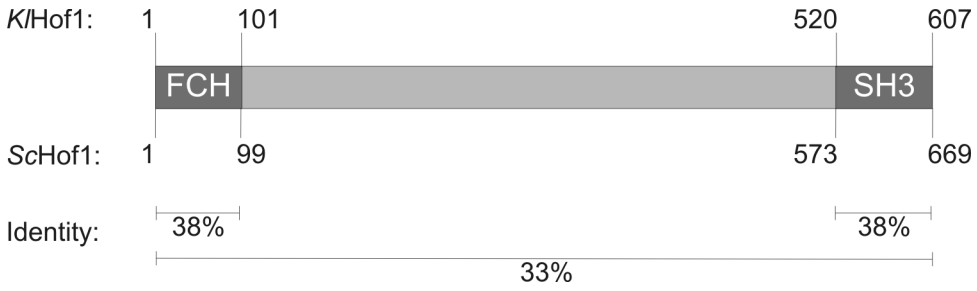


Fig. 3.7: *Kl*Hof1 and *Sc*Hof1 share 33% identity.
Both Hof1-homologues from *K. lactis* and *S. cerevisiae* have a similar modular arrangement, sharing 33% identity overall and 38% within the FCH- and SH3-domain. The schematic drawing indicates the respective spans by giving the first and last amino acids.

Both domains and the overall length of the protein are conserved in the *Kl*Hof1 homologue. An alignment between the deduced amino acid sequences from bakers and milk yeast displays 33% identity with slightly higher values within the SH3- and the FCH-domains (both 38%, see also Fig. 3.7). As exemplified above, a heterozygous deletion of the complete ORF was constructed in the diploid wild-type strain KHO60 to investigate the role of Hof1 for cytokinesis in *K. lactis*, resulting in strain KNH9 (*Klhof1::ScURA3/KlHOF1*). After sporulation, tetrads were dissected, and displayed a 2:0-segregation for viability (Fig. 3.8), which again indicates that the gene is essential. PCR analyses and replica-plating of the viable segregants indeed proved that they did not harbor the *Klhof1* deletion and that they all required uracil for growth.

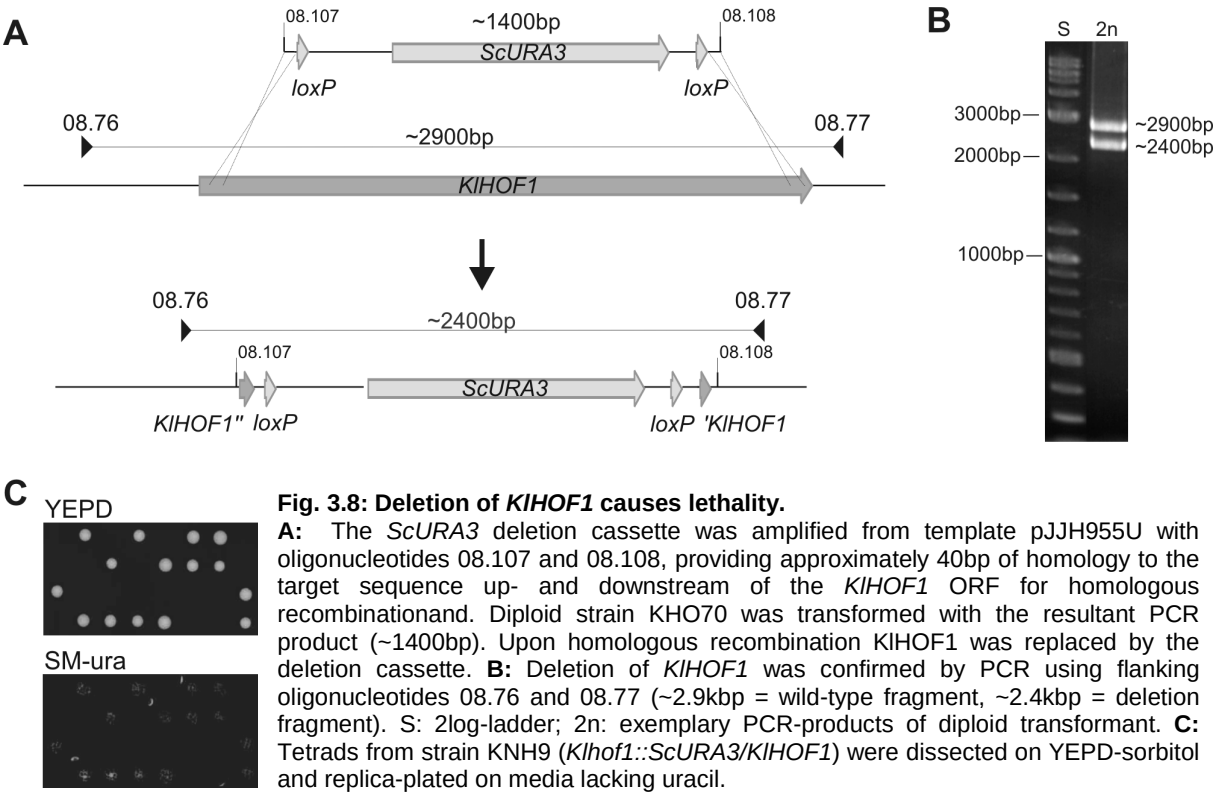


Fig. 3.8: Deletion of *KlHOF1* causes lethality.
A: The *ScURA3* deletion cassette was amplified from template pJJH955U with oligonucleotides 08.107 and 08.108, providing approximately 40bp of homology to the target sequence up- and downstream of the *KlHOF1* ORF for homologous recombinationand. Diploid strain KHO70 was transformed with the resultant PCR product (~1400bp). Upon homologous recombination KlHOF1 was replaced by the deletion cassette. **B:** Deletion of *KlHOF1* was confirmed by PCR using flanking oligonucleotides 08.76 and 08.77 (~2.9kbp = wild-type fragment, ~2.4kbp = deletion fragment). S: 2log-ladder; 2n: exemplary PCR-products of diploid transformant. **C:** Tetrads from strain KNH9 (*Klhof1::ScURA3/KlHOF1*) were dissected on YEPD-sorbitol and replica-plated on media lacking uracil.

Micrographs of wild-type and deletion segregants showed that spores from the non-viable segregants were merely able to undergo two cell divisions before ceasing proliferation (Fig. 3.9). In contrast, *Klhof1* disruption mutants (KNH7; *Klhof1::ScLEU2/Klhof1::ScLEU2*), which carried a marker insertion rather than a replacement within the *KlHOF1* ORF (Fig. 3.10A), were viable but displayed aberrant cell morphologies. This strain was subjected to Calcofluor white treatment in order to stain the chitin at the bud neck and of bud scars remaining on the mother cell after each cell division.

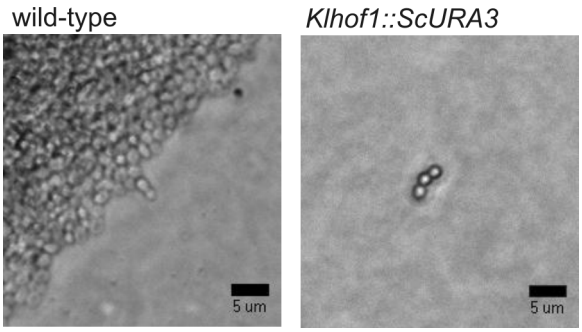


Fig. 3.9: Klhof1Δ mutants are defective in proliferation.
A wild-type and a deletion segregant of strain KNH9 (*Klhof1::ScURA3/KlHOF1*) were cut out from the tetrad plate and transferred to a glass slide. Micrographs were taken as DIC-images.

The cells formed chains with strong fluorescent signals at the bud necks, indicating that the primary septa were not degraded properly and abscission of the daughter cells failed (Fig. 3.10B). It can be concluded, that *Kl*Hof1 serves a more important role in cytokinesis in *K. lactis*, as compared to its homologue in baker's yeast, whose deletion is only temperature-sensitive.

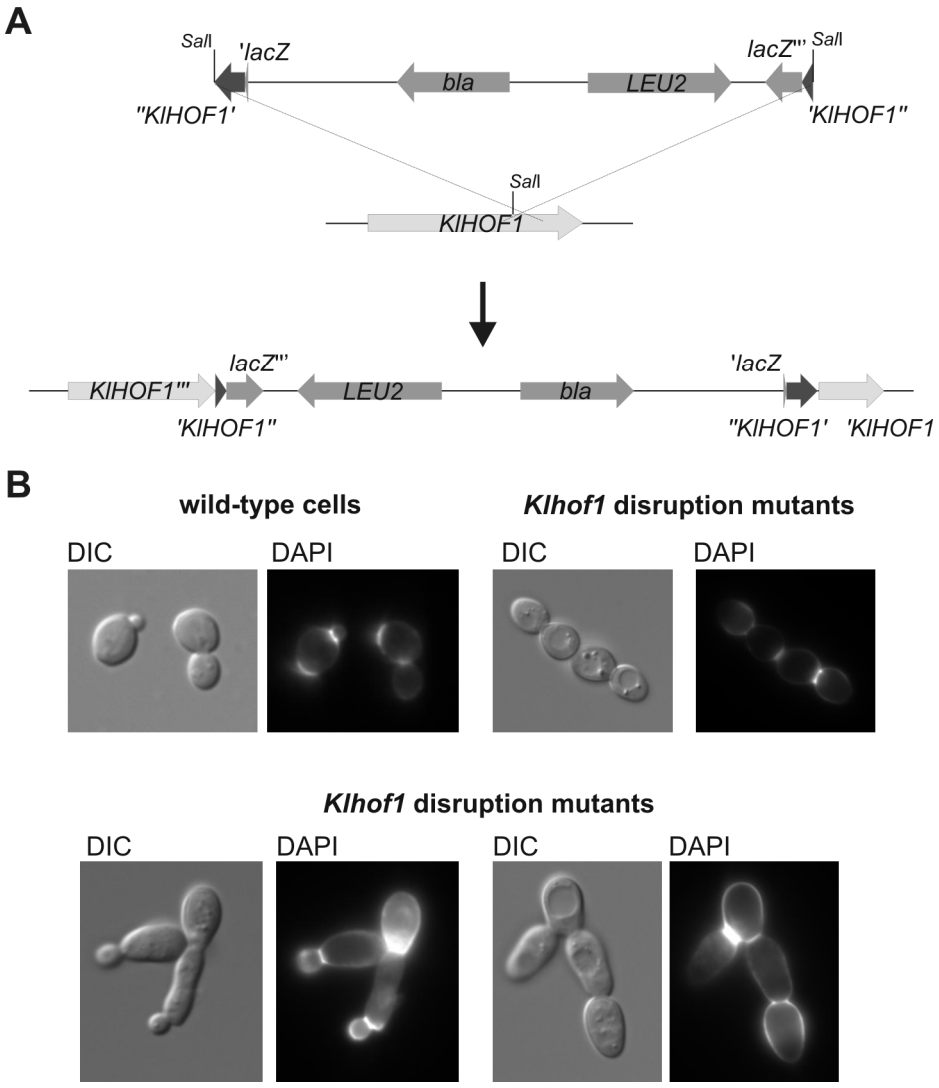


Fig. 3.10: Homozygous diploid disruption mutants of *Klhof1* display aberrant cell morphologies.
A: Plasmid pNH56 (YIplac128-*KlHOF1*$_{1012\text{-}1324}$) was linearized with enzyme *Sal*I and wild-type strain KHO60 was transformed, resulting in a *Klhof1* disruption strain. **B:** Wild-type cells and cells from strain KNH7 (*Klhof1::ScLEU2/Klhof1::ScLEU2*) were subjected to CFW staining (2.2.10.4) and fluorescent images were taken through the DAPI filter.

3.2.1.3 *Kl*Inn1 is an essential protein

*Sc*Inn1 is an essential protein, which mediates the ingression of the plasma membrane during cytokinesis and thus triggers the separation of the cytoplasmata of mother and daughter cell. It contains a C2-domain at its N-terminus, which presumably interacts with membrane lipids, as well as several conserved proline-rich motifs in its C-terminal half. *Scinn1* null mutants show severe cytokinesis defects and do not produce viable progeny. The protein shares 35% amino acid identity with its homologue from *K. lactis*, with higher identities if only the C2-domains are aligned (51%; Fig. 3.11).

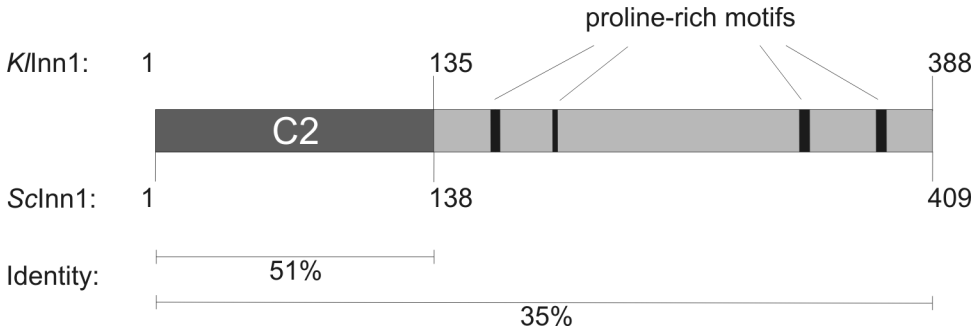


Fig. 3.11: *Kl*Inn1 and *Sc*Inn1 share 35% identity.
*Kl*Inn1 and *Sc*Inn1 have a similar modular arrangement, sharing 35% identity overall and 51% within the C2-domain. The schematic drawing indicates the respective spans by giving the first and last amino acids.

In order to gain some insight into the *in vivo* function of *Kl*Inn1, a heterozygous diploid *Klinn1* deletion strain was constructed (KNH10; *Klinn1::ScLEU2/KlINN1*) and subjected to tetrad analysis. As described for the two genes investigated above, a 2:0-segregation for spore viability was observed in a total of eight tetrads analyzed (Fig. 3.12). The viable segregants invariably required leucine for growth, demonstrating that the non-viable spores harbor the *Klinn1* deletion. Microscopical examination revealed that the non-viable segregants initially germinated and were able to produce a few daughter cells, which however failed to separate from the mother cells. It can be concluded that *KlINN1* is essential for proper cytokinesis in *K. lactis,* as it is in *S. cerevisiae*.

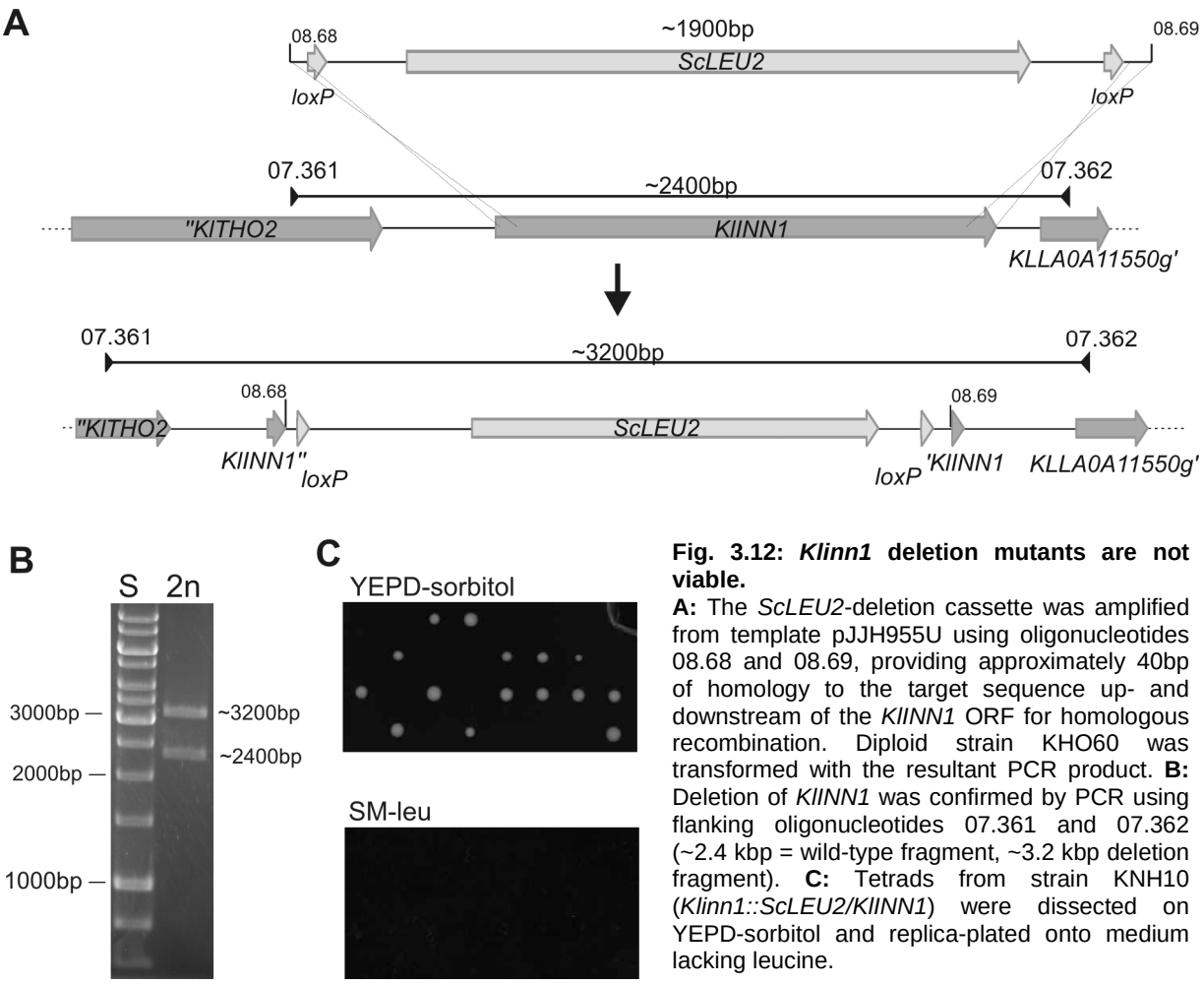


Fig. 3.12: *Klinn1* deletion mutants are not viable.
A: The *ScLEU2*-deletion cassette was amplified from template pJJH955U using oligonucleotides 08.68 and 08.69, providing approximately 40bp of homology to the target sequence up- and downstream of the *KlINN1* ORF for homologous recombination. Diploid strain KHO60 was transformed with the resultant PCR product. **B:** Deletion of *KlINN1* was confirmed by PCR using flanking oligonucleotides 07.361 and 07.362 (~2.4 kbp = wild-type fragment, ~3.2 kbp deletion fragment). **C:** Tetrads from strain KNH10 (*Klinn1::ScLEU2/KlINN1*) were dissected on YEPD-sorbitol and replica-plated onto medium lacking leucine.

3.2.1.4 Lack of *Kl*Myo1 causes heat sensitivity in *K. lactis*

Myo1 is a central component of the contractile actomyosin ring (CAR) necessary for cytokinesis in *S. cerevisiae.* Moreover, fusions of Myo1 with fluorescent proteins are a valuable tool to follow the kinetics of the AMR constriction and thus the timing of cytokinesis (Jendretzki et al., 2009). Although a similar function may be presumed for all yeasts, the Myo1 homologue of *K. lactis* has not yet been investigated. Alignment of the deduced amino acid sequences of *Kl*Myo1 and *Sc*Myo1 showed an overall identity of 44%. Especially near the N-terminus, some regions are 90-100 % identical (Fig. 3.13).

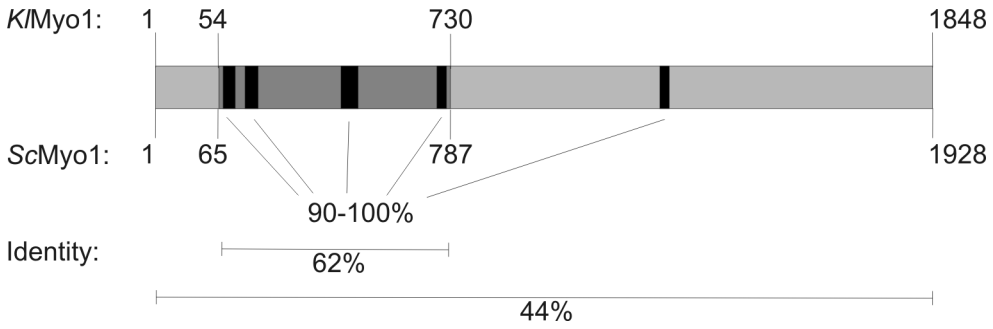


Fig. 3.13: *Kl*Myo1 and *Sc*Myo1 share 44% identity.
*Kl*Myo1 and *Sc*Myo1 comprise the type II myosin heavy chain. The amino acid sequences of both homologues display 44% overall identity with an higher amount in the N-terminal half (62%, dark gray) and up to 100% in short areas (black).The schematic drawing indicates the respective spans by giving the first and last amino acids.

In a first comparative approach, a heterozygous diploid deletion strain was constructed (DSA01; *Klmyo1::KanMX/KlMYO1*, Fig. 3.14) and subjected to tetrad analysis. All tetrads investigated produced four viable segregants, indicating that *KlMYO1* is not an essential gene in contrast to the three cytokinesis regulators investigated above and to its *S. cerevisiae* homologue in most strain backgrounds.

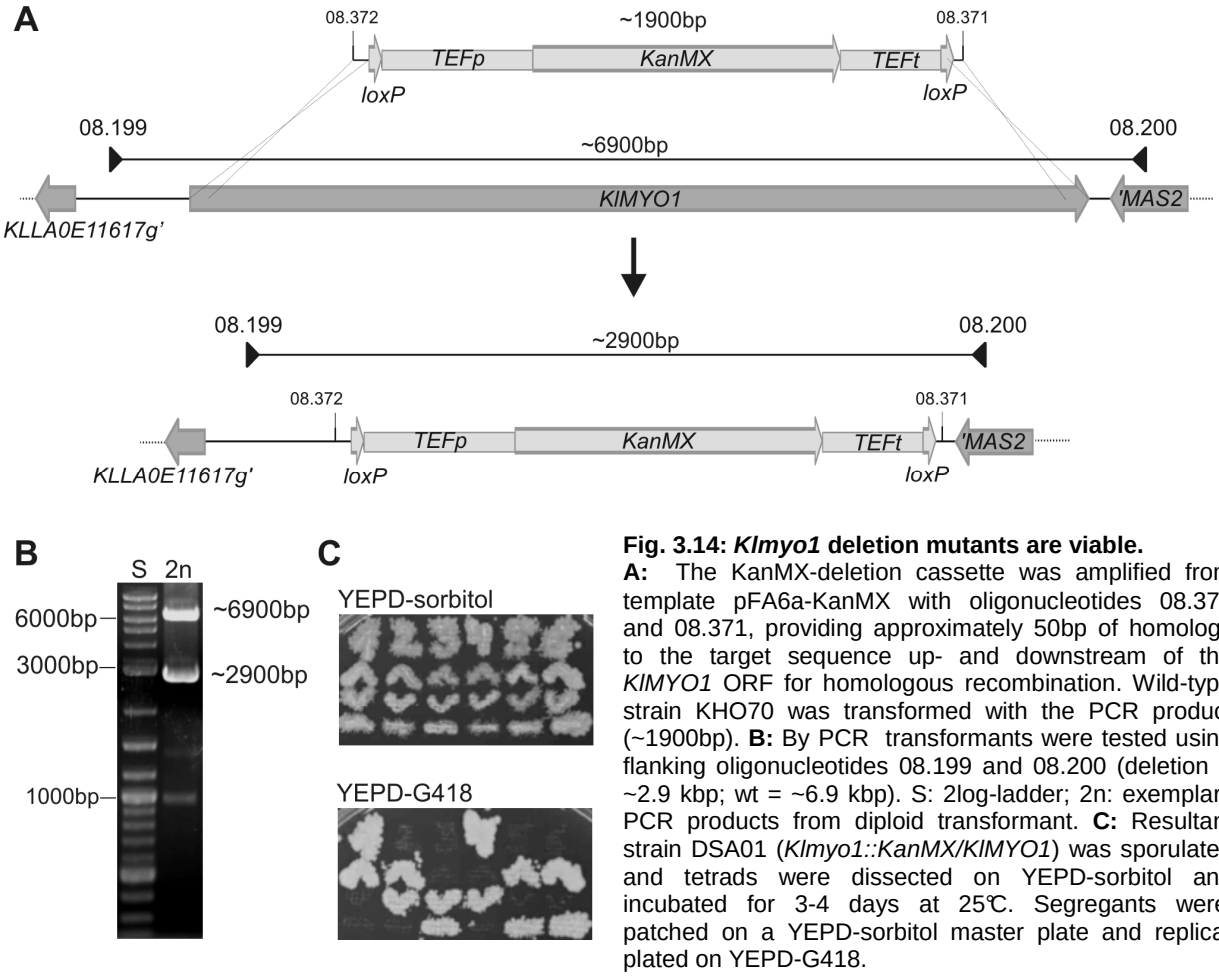


Fig. 3.14: *Klmyo1* deletion mutants are viable.
A: The KanMX-deletion cassette was amplified from template pFA6a-KanMX with oligonucleotides 08.372 and 08.371, providing approximately 50bp of homology to the target sequence up- and downstream of the *KlMYO1* ORF for homologous recombination. Wild-type strain KHO70 was transformed with the PCR product (~1900bp). **B:** By PCR transformants were tested using flanking oligonucleotides 08.199 and 08.200 (deletion = ~2.9 kbp; wt = ~6.9 kbp). S: 2log-ladder; 2n: exemplary PCR products from diploid transformant. **C:** Resultant strain DSA01 (*Klmyo1::KanMX/KlMYO1*) was sporulated and tetrads were dissected on YEPD-sorbitol and incubated for 3-4 days at 25℃. Segregants were patched on a YEPD-sorbitol master plate and replica-plated on YEPD-G418.

To further characterize the deletion phenotype, a drop dilution assay with segregants of two complete tetrads was performed on different media at variable temperatures.

The deletion mutants grew similar to the wild-type segregants on YEPD, YEPD plus sorbitol or SC-medium, indicating that the lack of *Kl*Myo1 does not require osmotic stabilization. Upon heat stress at 37 ℃, only cells expressing wild-type *KlMYO1* showed some residual growth, whereas *Klmyo1* deletion mutants were not able to grow at all (Fig. 3.15).

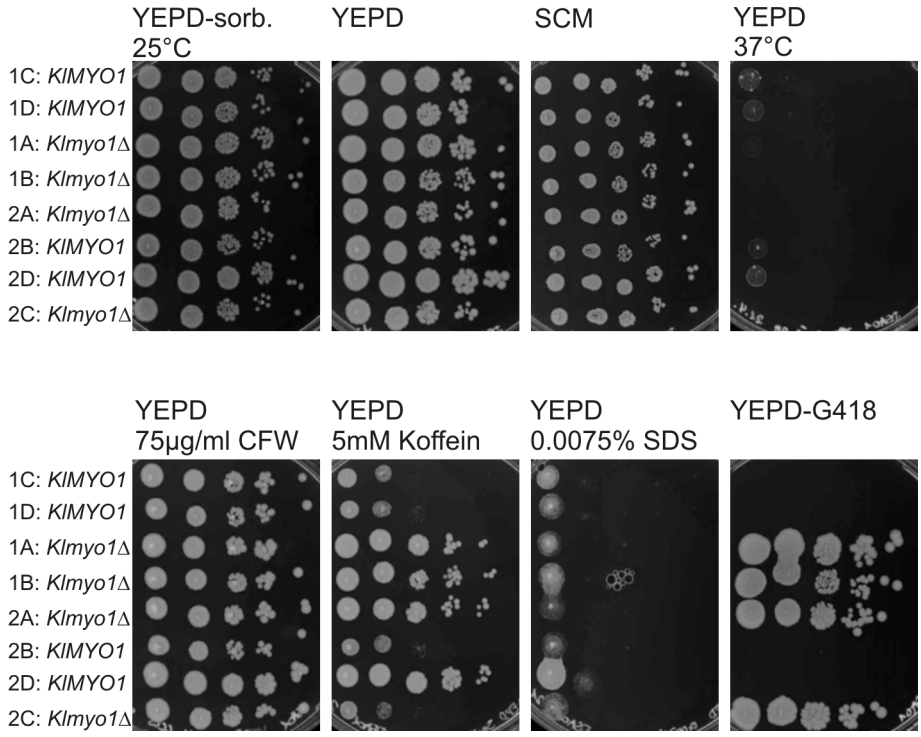


Fig. 3.15: Haploid *Klmyo1*-deletion mutants display slightly increased heat sensitivity.
Segregants of two tetrads originating from strain DSAO1 (*Klmyo1::KanMX/KlMYO1*) were tested in serial drop dilution assays as described in 2.2.8. Plates were incubated for 4 days at 30℃ or as indicated.

Several cell wall perturbing agents were also tested in these drop dilution assays. With 75 µg/ml Calcofluor white (CFW) all cells showed a similar growth behavior to the wild type, indicating that loss of *KlMYO1* does not affect the organization of chitin within the cell wall. A similar result was obtained on plates with 0.0075% SDS, where only the drops with the highest cell densities grew. In contrast, four of the eight tested segregants displayed a retarded growth on plates containing 5 mM caffeine. However, this phenotype was independent of the *KlMYO1* allele, but rather segregated with the *KlADE2* locus. Thus, strains which were sensitive against caffeine carried the *Klade2* deletion, whereas the resistant strains were wild-type. A similar phenotype for *ade2* mutants has been reported for *S. cerevisiae* and suggested to be due to a competitive inhibition of the adenine transporter by caffeine (Bard et al., 1980).

The temperature-sensitive phenotype of *Klmyo1* deletions was further investigated by time lapse analyses of growth under the microscope. Wild-type cells initially did not show any severe defects at higher temperatures (37°C), but some cells ceased proliferation after approximately 6h of incubation. In contrast, the *Klmyo1* deletion mutants displayed abnormalities in cell separation approximately five hours after the shift to the higher temperature, resulting in elongated cells and the formation of cell chains (data not shown). To further analyze these findings, cells were incubated at different temperatures and stained with CFW to determine the distribution of chitin within their cell walls and subjected to fluorescence microscopy (Fig. 3.16).

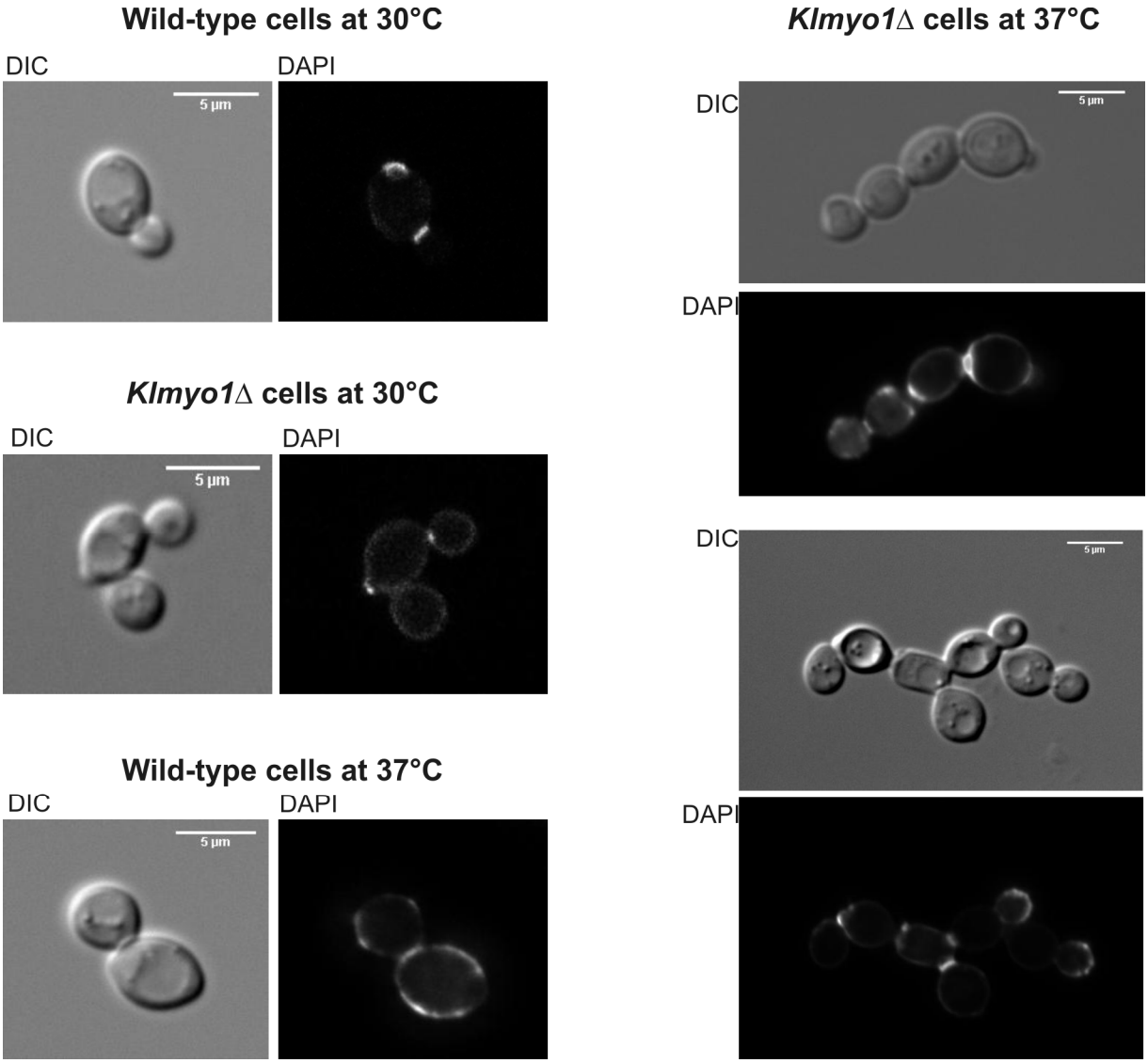


Fig. 3.16: Upon heat stress *Klmyo1* deletion mutants form cell chains.
Wild-type and *Klmyo1* deletion segregants (DSA01) were subjected to CFW stainining (see section 2.2.10.4) and fluorescence microscopy after incubation at indicated temperatures for three hours. Emission was detected through the DAPI-filter.

Neither bud scars nor the primary septa displayed any abnormal morphologies in *Klmyo1* deletions as compared to the wild-type control cells when incubated at 30℃.

In contrast, incubation at 37℃ revealed that the cells lacking *KlMYO1* formed chains, multi-budded mother cells and daughter cells producing buds themselves, before being separated from the mother cell. This further supports the view that *Kl*Myo1 is involved in the constriction of the AMR in *K. lactis*, but indicates that it is far less important for cytokinesis in *K. lactis*, than it is in *S. cerevisiae*.

3.2.2 Complementation analyses

3.2.2.1 *Kl*Cyk3

In bakers yeast, *Sc*Cyk3 is an important protein for AMR-independent cytokinesis (Jendretzki et al., 2009) (Nishihama et al., 2009). Whereas a *Sccyk3* deletion is perfectly viable and does not show any pronounced phenotype, it is synthetically lethal with a *Schof1* deletion. To further characterize the role of *Kl*Cyk3, the heterozygous deletion mutant of the encoding gene constructed above (see 3.2.1.1) was employed in complementation analyses with *ScCYK3*. The latter gene was introduced on a centromeric plasmid (pNH94; pCXJ20-*ScCYK3*) into the heterozygous recipient strain (KHN8), which was then subjected to sporulation and tetrad analysis (Fig. 3.17). Although all of the tetrads displayed a 2:0-segregation, it could not be assumed, that *ScCYK3* is not able to complement lack of *KlCYK3*, since only two out of the 18 viable segregants carried the plasmid. To avoid the chance of plasmid loss during meiosis, integrative plasmids were constructed, enabling integration of *ScCYK3* either at the deletion locus of *Klcyk3::ScURA3* or at the native *Klleu2,3-112* locus. Unfortunately, this approach did not succeed either as well as did not mass spore analysis (see section 2.2.3).

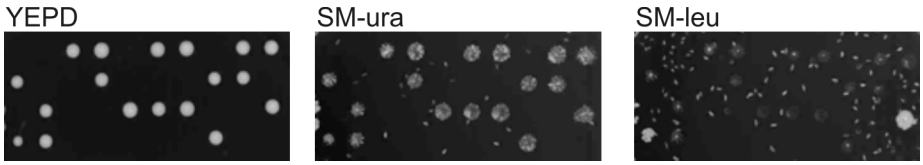


Fig. 3.17: The complementation ability of *ScCYK3* in *Klcyk3*Δ mutants could not be analysed.
KNH8 (*Klcyk3::ScURA3/KlCYK3*) was transformed with plasmid pNH94 (pCXJ20-*ScCYK3*) and subjected to tetrad analysis on a YEPD-plate. After incubation for 3-4 days, plate was replica-plated on SM-ura and SM-leu plates.

Vice versa, *Kl*Cyk3 was introduced on the plasmid pNH92 (pCXJ22-*KlCYK3*) into a heterozygous diploid strain which harbors deletions of the SH3-domains of both *Sc*Cyk3 and *Sc*Hof1 (DAJ34; *ScCYK3ΔSH3-3HA::SpHIS3/ScCYK3 ScHOF1ΔSH3-3HA::KanMX/ScHOF1*), in order to test its ability to complement the lethality of a

haploid SH3 double deletion after tetrad analysis. Thus, haploid strains carrying the double deletion and the plasmid would only be viable, if the *K. lactis* homologue can functionally complement in *S. cerevisiae*. This was indeed the case (Fig. 3.18), since strains being phototrophic for both uracil and histidine, as well as resistant to G418 were obtained within five out of seven tetrads investigated. The complementation capacity of the plasmid-encoded *KlCYK3* was further confirmed by counter selection with 5-fluoroorotic acid (5-FOA), which only affects cells with a functional *URA3* allele. Since the latter was also exclusively carried on the plasmid introduced, none of the transformants also carrying the *Sccyk3 Schof1* SH3 double deletion survived on 5-FOA plates.

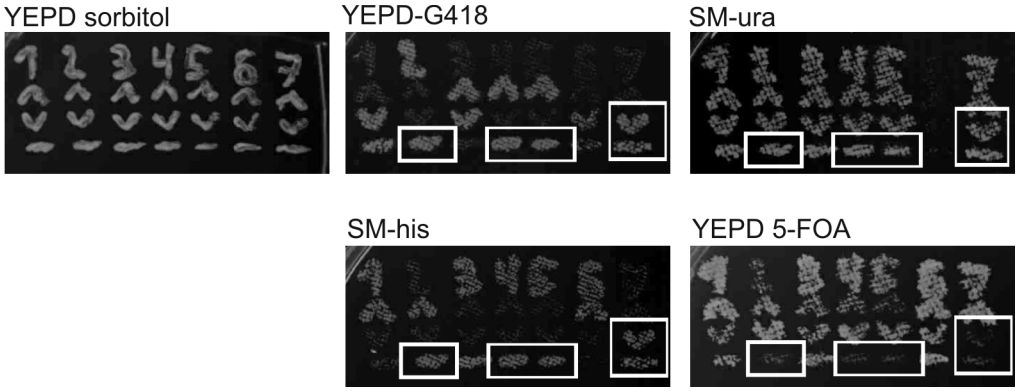


Fig. 3.18: Expression of *KlCYK3* restores viability to *ScCYK3ΔSH3 ScHOF1ΔSH3* double deletion mutants. Strain DAJ34 (*ScCYK3ΔSH3-3HA::SpHIS3/ScCYK3 ScHOF1ΔSH3-3HA::KanMX/ScHOF1*) was transformed with plasmid pNH122 (pCXJ22-*KlCYK3*, *URA3*) and subjected to tetrad analysis. Segregants were patched and replica-plated.

3.2.2.2 *Kl*Hof1

As shown above, a deletion of *HOF1* in *K. lactis* is lethal, whereas lack of its homologue in *S. cerevisiae* merely causes heat sensitivity and synthetic lethality with *Sccyk3*. Thus, similar experiments to the ones described in the preceding chapter were performed to investigate *KlHOF1*.

First, the diploid *K*. *lactis* strain KNH9 heterozygous for the *Klhof1* deletion was used as a recipient to introduce the *ScHOF1* gene on a centromeric vector. To select for the respective plasmid and to allow for its subsequent counter selection on 5-FOA, the *ScURA3* allele used for constructing the *Klhof1* deletion had first to be eliminated. This was achieved by introducing a plasmid-encoded Cre-recombinase on the vector pJJH959 with selection for leucine prototrophy, inducing gene expression by growth on lactose medium and screening for a strain that required both uracil and leucine for growth, i.e. having lost the chromosomal *ScURA3* cassette by recombination

between the flanking *loxP* sites and the Cre-recombinase plasmid by mitotic segregation (see 2.2.7). The resulting strain KNH19 was used to introduce *ScHOF1* on plasmid pNH120 (pCXs22-*ScHOF1*) and subjected to sporulation and tetrad analysis. Unfortunately, only three segregants out of 38 tetrads displayed prototrophy for uracil, but were proven to be diploid by PCR. Due to the statistically low reliability of this approach, the complementation capacity of *ScHOF1* was investigated by mass spore analysis (see 2.2.3). In order to deplete the amount of vegetative cells and thus to enrich for spores, the starved transformants were incubated with Zymolyase for one hour and directly spread on selective plates instead of being dissected for tetrad analysis. Replica-plating and PCR analyses revealed that some segregants carried a single *loxP* site at the *KlHOF1* locus, and that growth on uracil depended on the presence of the pCXs22-*ScHOF1* plasmid (Fig. 3.19). Thus, replica-plating on 5-FOA plates and depletion of the plasmid resulted in loss of viability in such segregants, indicating that *Sc*Hof1 can functionally substitute *Kl*Hof1.

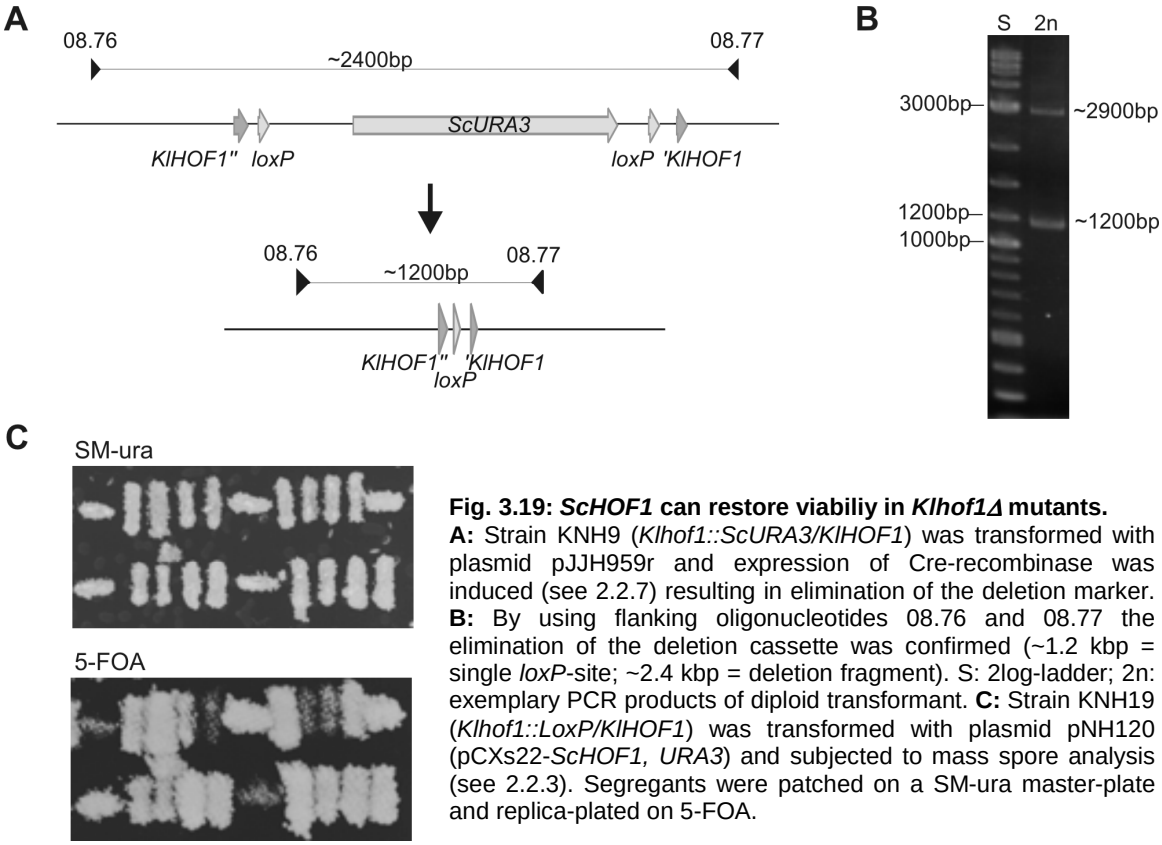


Fig. 3.19: *ScHOF1* can restore viabiliy in *Klhof1Δ* mutants.
A: Strain KNH9 (*Klhof1::ScURA3/KlHOF1*) was transformed with plasmid pJJH959r and expression of Cre-recombinase was induced (see 2.2.7) resulting in elimination of the deletion marker. **B:** By using flanking oligonucleotides 08.76 and 08.77 the elimination of the deletion cassette was confirmed (~1.2 kbp = single *loxP*-site; ~2.4 kbp = deletion fragment). S: 2log-ladder; 2n: exemplary PCR products of diploid transformant. **C:** Strain KNH19 (*Klhof1::LoxP/KlHOF1*) was transformed with plasmid pNH120 (pCXs22-*ScHOF1, URA3*) and subjected to mass spore analysis (see 2.2.3). Segregants were patched on a SM-ura master-plate and replica-plated on 5-FOA.

For complementation studies in *S. cerevisiae*, again the heterozygous diploid strain DAJ34 carrying *ScCYK3-SH3 ScHOF1-SH3* deletions (*ScCYK3ΔSH3-3HA::SpHIS3/ScCYK3 ScHOF1ΔSH3-3HA::KanMX/ScHOF1*) was used to introduce

the *KlHOF1* gene on a centromeric plasmid (pNH110, pCXJ22-*KlHOF1*) selecting for its *URA3* marker. In the following tetrad analysis, mostly all four segregants formed colonies, suggesting that expression of *KlHOF1* restores viability to the double deletion segregants (Fig. 3.20). This was further confirmed by the ability of such segregants to grow in the absence of uracil (plasmid marker) and histidine, as well as in the presence of G418 (deletion markers). Double deletions carrying the heterologous gene were not viable on 5-FOA plates, as expected.

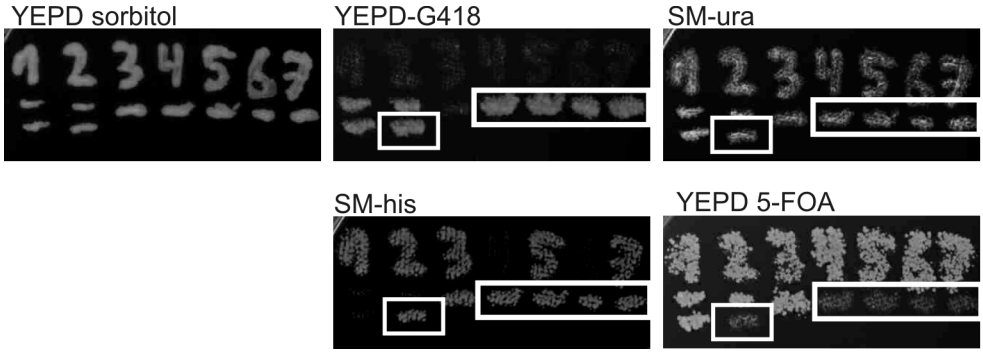


Fig. 3.20: Expression of *KlHOF1* represses lethality of *ScCYK3-SH3 ScHOF1-SH3* double deletion mutants. Strain DAJ34 (*ScCYK3ΔSH3-3HA::SpHIS3/ScCYK3 ScHOF1ΔSH3-3HA::KanMX/ScHOF1*) was transformed with plasmid pNH110 (pCXJ22-*KlHOF1*, *URA3*) and subjected to tetrad analysis. Segregants were patched and replica-plated. Frames mark double deletion segregants harboring the plasmid.

3.2.2.3 *Kl*Inn1

Haploid strains with deletions of *INN1* are not viable in either *S. cerevisiae* or *K. lactis*. For further comparison of the respective protein functions, again cross-species complementation analyses were carried out. Thus, the heterozygous diploid deletion strain KNH10 (*Klinn1::ScLEU2/KlINN1*) was transformed with pNH76 which carries the *ScINN1* gene inserted into the vector pCXJ18. A subsequent tetrad analysis produced only two viable segregants each from a total of 17 tetrads analyzed, indicating that *ScINN1* cannot complement the growth defect of a *Klinn1* deletion (Fig. 3.21). Replica-plating and PCR analyses confirmed that all viable segregants exclusively carried a wild-type allele of *KlINN1* at the respective chromosomal locus (data not shown).

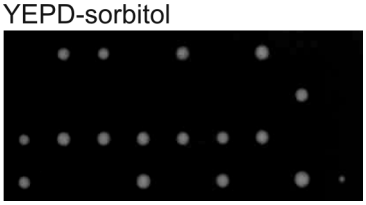


Fig. 3.21: Expression of *ScINN1* does not complement lack of *Klinn1*. Strain KNH10 (*Klinn1::ScLEU2/KlINN1*) was transformed with plasmid encoded *ScINN1* under the control of its own promoter (pNH76, pCXJ22-*ScINN1*, *URA3*) and subjected to tetrad analysis. Segregants were incubated for 3-4 days at 28°C.

To test if *Kl*Inn1 can complement the *inn1* deletion of *S. cerevisiae*, strain DAJ24 (*Scinn1::SpHIS3/ScINN1*) was used as a recipient for a plasmid encoded copy of *KlINN1* (pJJH1008, YEp352-*KlINN1*). A subsequent tetrad analysis similar to the one performed above in *K. lactis* confirmed that the *KlINN1* gene is also not able to complement the respective null mutant in *S. cerevisiae* (Fig. 3.22). The lack of complementation between the two yeast species could be attributed to a low-level expression of *KlINN1* in *S. cerevisiae*, since the gene was introduced under the control of its native *K. lactis* promoter. To exclude this possibility, the native promoter was replaced by the fairly strong glycolytic *ScPFK2* promoter in plasmid pJJH1013 (YEp352-*ScPFK2p-KlINN1*). Introduction of this plasmid into DAJ24 and subsequent tetrad analysis again displayed a 2:0-segregation for viability, confirming that *KlINN1* cannot complement for its *S. cerevisiae* homologue, even if its expression is driven by a native *S. cerevisiae* promoter.

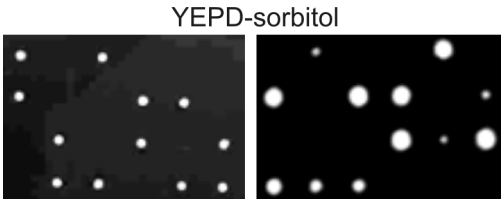


Fig. 3.22: Expression of *KlINN1* is not able to restore viability to a haploid *Scinn1* deletion strain.
Strain DAJ24 (*Scinn1::SpHIS3/ScINN1*) was transformed with plasmids encoding *KlINN1* under control of either its own promoter (pJJH1008, left panel) or that from *ScPFK2* (pJJH1013, right panel). Transformants were subjected to tetrad analyses and plates were incubated for 3-4 days at 25°C.

Given the fact that comparisons of the primary sequences indicated a conservation of the functional domains (i.e. the C2-domain and the relevant proline-rich motifs), this result was surprising. In order to address the question which parts of the Inn1-homologues may be responsible for this apparent species-specificity, hybrid proteins were constructed, with an exchange of the N-terminal part comprising the C2-domains of *Kl*Inn1 and *Sc*Inn1. These constructs were introduced on a centromeric shuttle vector suitable for expression both in *K. lactis* and in *S. cerevisiae*. For further analyses, the strains and strategies as described above for the heterologous complementation studies were employed.

As evident from Fig. 3.23, hybrid proteins carrying the C2-domain of *Kl*Inn1 complemented the *Klinn1* deletion, but not the *ScInn1* deletion. *Vice versa,* Inn1 proteins with the C2-domain derived from *S. cerevisiae* complemented the *Scinn1*, but not the *Klinn1* defect. In conclusion, the C2-domain seems to determine the species-specificity of Inn1, suggesting a difference in its cellular interaction partners present in the two yeasts.

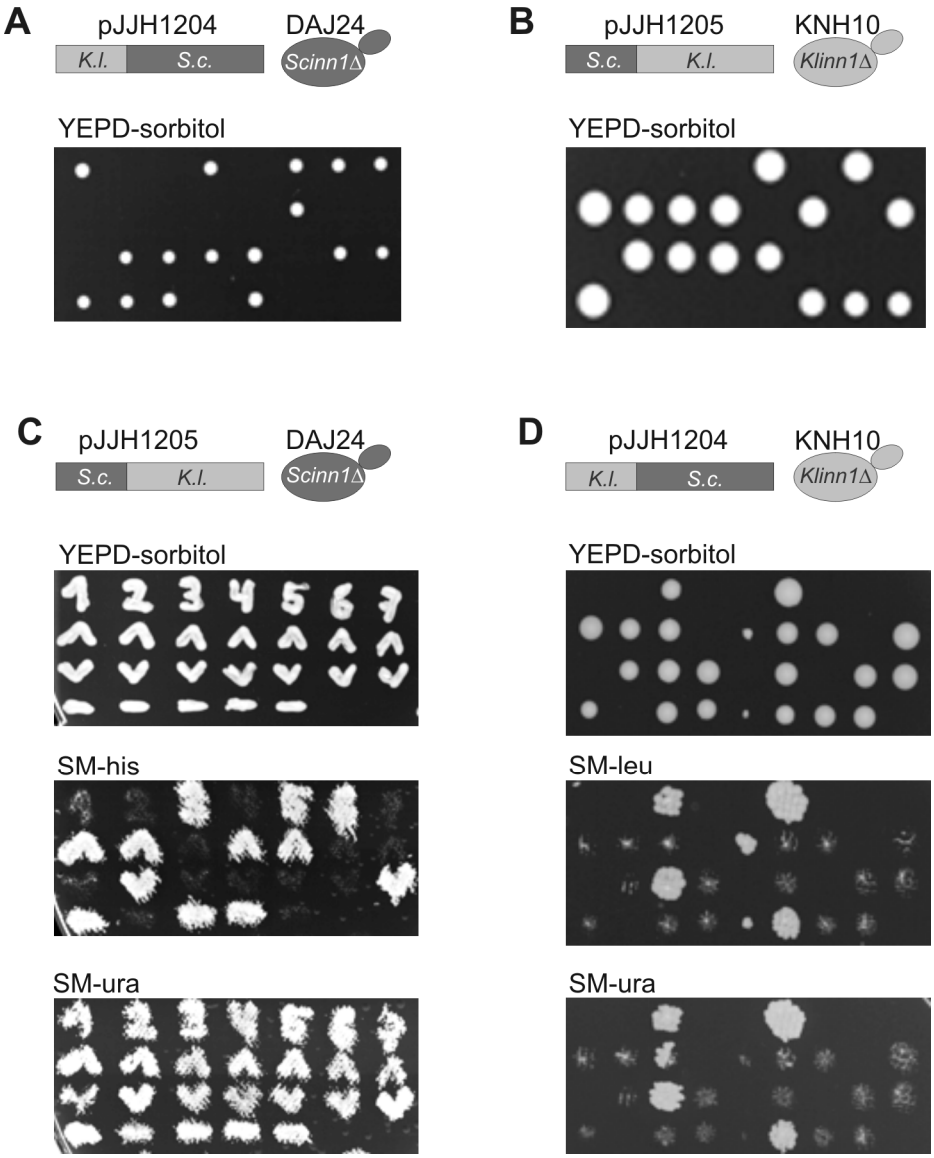


Fig. 3.23: The C2-domain determines species-specificity of Inn1 in both homologues.
A: Strain DAJ24 (*Scinn1::SpHIS3/ScINN1*) was transformed with the plasmid pJJH1204, encoding the hybrid *KlINN1*$_{C2}$-*ScINN1*$_{PXXP}$ on vector YEP352 (*URA3*), and subjected to tetrad analysis on YEPD-sorbitol plates. **B:** After transformation of strain KNH10 (*Klinn1::ScLEU2/KlINN1*) with the hybrid-plasmid pJJH1205 (*ScINN1*$_{C2}$-*KlINN1*$_{PXXP}$-pCXS22, *URA3*), sporulation was induced and tetrads were dissected on YEPD-sorbitol plates. **C:** Tetrads of strain DAJ24 carrying hybrid-construct pJJH1205 were dissected on YEPD-sorbitol plates. Segregants were replica-plated on SM-his and SM-ura plates. **D:** Strain KNH10 was employed as recipient for plasmid pJJH1204 and transformants were subjected to tetrad analysis on YEPD-sorbitol. Resultant segregants were replica-plated on SM-leu and SM-ura plates.

3.2.2.4 *Kl*Myo1

Attempts to investigate the complementation capacity of *ScMYO1* in the respective haploid deletion mutant of *K. lactis* (see above, section 3.2.1.4) failed, since the temperature-sensitive growth defect could not even be complemented by expression of the homologous *KlMYO1* gene introduced on a plasmid. Thus, the similar lack of complementation by the *ScMYO1* gene cannot be interpreted as a lack of function and would need further investigation (data not shown).

On the other hand, the *Scmyo1* deletion shows a severe growth defect at 37°C, which can be fully complemented by expression of a plasmid-encoded copy of *ScMYO1* (pSA15, pCXs22-*ScMYO1*; Fig. 3.24). In contrast, a similar vector carrying the *KlMYO1* gene (pSA12, pCXs22-*KlMYO1*) does not confer growth to this mutant at the elevated temperature. In analogy to Inn1, this result suggests that Myo1 exerts species-specific functions in the two yeasts and lacks the ability for heterologous complementation.

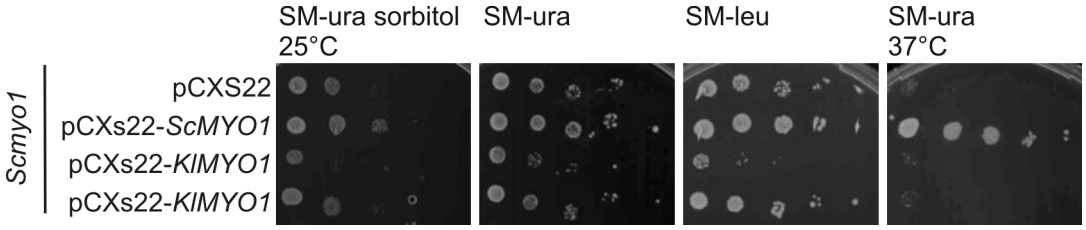


Fig. 3.24: Expression of *KlMYO1* can not suppress heat sensitivity of *Scmyo1* deletion mutants.
Strain HAJ37 (*Scmyo1::SpLEU2*) was transformed with vector pCXS22 (*URA3*) and derivatives carrying *ScMYO1* (pSA15) or *KlMYO1* (pSA12), respectively. Transformants were subjected to serial drop dilution assays as described in 2.2.8 and incubated for 3 days at 30°C or as indicated.

3.2.3 Localization studies

The *S. cerevisiae* homologues of the four proteins discussed in the previous sections are all involved in cytokinesis and consequently localize to the yeast bud neck at specific times during this process. Moreover, the cross-species complementation of the Cyk3 and Hof1 proteins reported above indicates a similar function in both yeasts, whereas the lack of complementation by Inn1 and Myo1 raised some doubts about their function in *K. lactis.* In the following, the intracellular localization of the *K. lactis* homologues tagged with fluorescence markers was therefore investigated.
Therefore, the GFP-coding sequence was fused to the 3'-end of the *KlCYK3* gene at its chromosomal locus in the diploid recipient strain KNH14 (*KlCYK3-GFP-KanMX/KlCYK3*). After verification of the correct fusion event in one of the *KlCYK3* alleles (Fig. 3.25), the respective strain was sporulated and subjected to tetrad analysis. The fact that all four spores produced viable progeny in the majority of the seven tetrads separated, indicates that the GFP fusion does not significantly affect *Kl*Cyk3 function (since the null mutant is lethal; see section 3.2.1). Fluorescence microscopy showed that the fusion protein locates to the bud neck in large budded cells of *K. lactis*, similar to the localization of its homologue in *S. cerevisiae*. Additional time-lapse analyses revealed that the signal of *Kl*Cyk3-GFP was

detectable for about 10 min at the bud neck in *K. lactis*, whereas *Sc*Cyk3-GFP is detected for 15 min at the *S. cerevisiae* bud neck during cytokinesis.

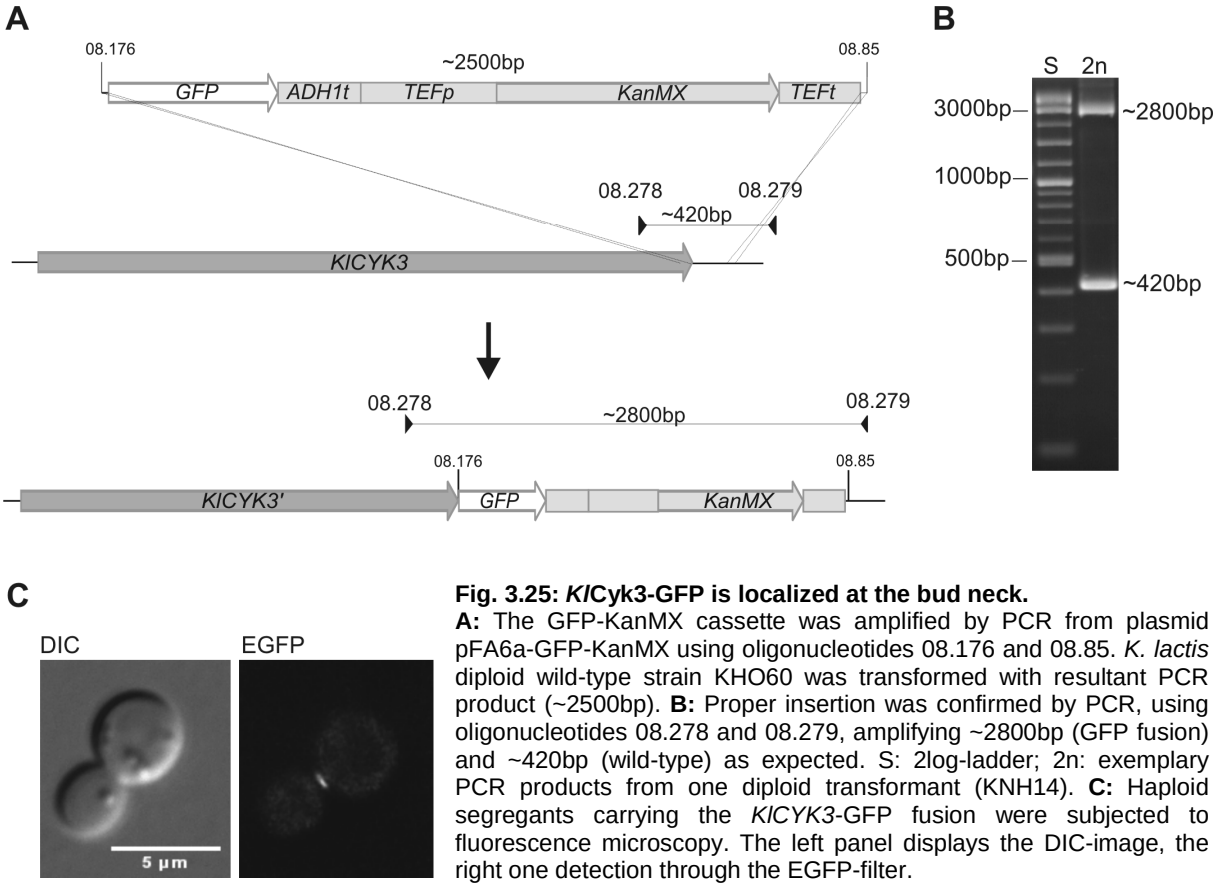


Fig. 3.25: *Kl*Cyk3-GFP is localized at the bud neck.
A: The GFP-KanMX cassette was amplified by PCR from plasmid pFA6a-GFP-KanMX using oligonucleotides 08.176 and 08.85. *K. lactis* diploid wild-type strain KHO60 was transformed with resultant PCR product (~2500bp). **B:** Proper insertion was confirmed by PCR, using oligonucleotides 08.278 and 08.279, amplifying ~2800bp (GFP fusion) and ~420bp (wild-type) as expected. S: 2log-ladder; 2n: exemplary PCR products from one diploid transformant (KNH14). **C:** Haploid segregants carrying the *KlCYK3*-GFP fusion were subjected to fluorescence microscopy. The left panel displays the DIC-image, the right one detection through the EGFP-filter.

In an analogous approach, the *KlHOF1* gene was also equipped with the coding sequence for GFP at its 3'-end at its chromosomal locus. The GFP-fusion cassette was introduced into the diploid *K. lactis* strain KHO70 and correct integration was confirmed by PCR (Fig. 3.26). After sporulation and tetrad analysis, all four spores were shown to produce viable progeny, with cells not displaying any aberrant morphology in microscopical examinations. Thus, it can be assumed that the *Kl*Hof1-GFP fusion protein is fully functional *in vivo*. Segregants carrying this GFP fusion were again subjected to fluorescence microscopy and revealed a specific GFP-signal at the bud neck of large budded cells. Since a similar localization was observed for its homologue in *S. cerevisiae*, and in the light of its capacity for heterologous complementation (see section 3.2.2.2), this indicates that *Kl*Hof1 serves a similar function in the regulation of cytokinesis in *K. lactis*, as does *Sc*Hof1 in *S. cerevisiae*.

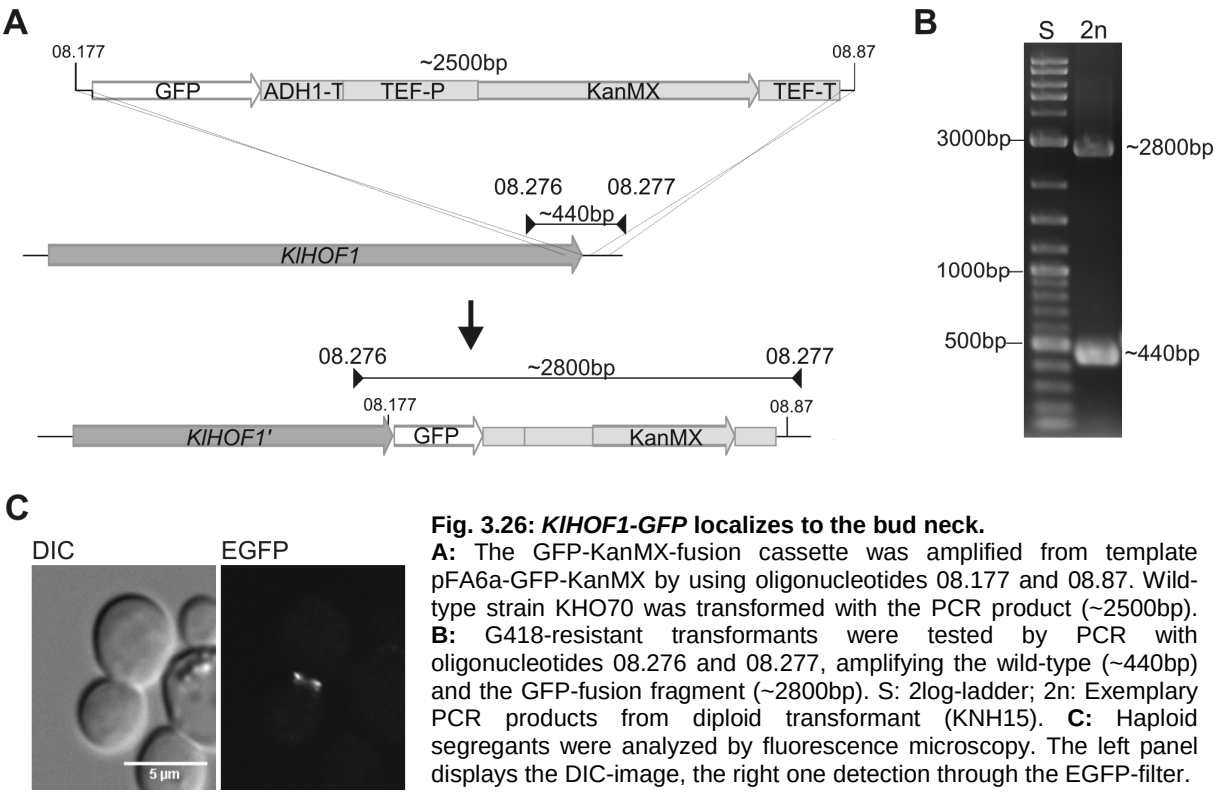
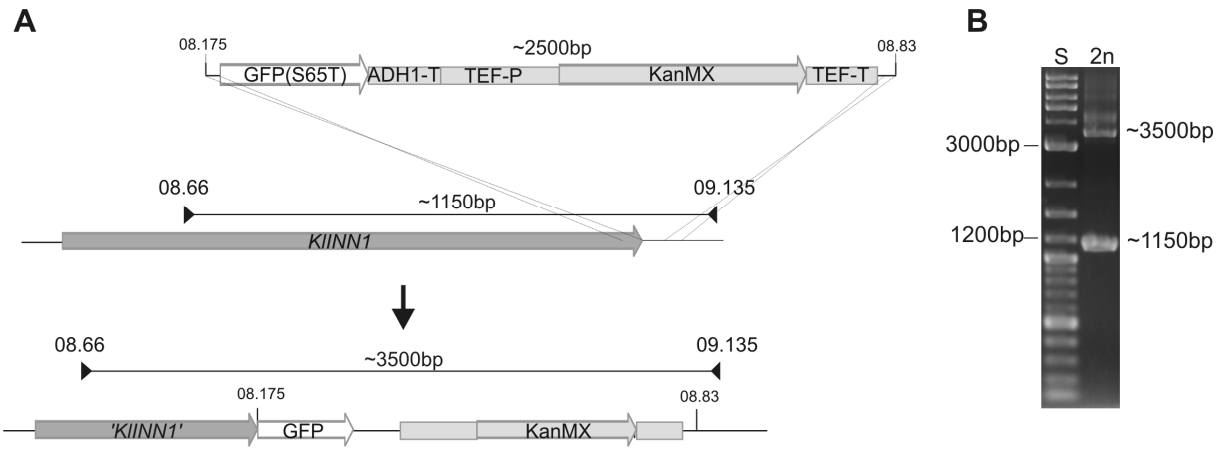


Fig. 3.26: *KlHOF1-GFP* localizes to the bud neck.
A: The GFP-KanMX-fusion cassette was amplified from template pFA6a-GFP-KanMX by using oligonucleotides 08.177 and 08.87. Wild-type strain KHO70 was transformed with the PCR product (~2500bp). **B:** G418-resistant transformants were tested by PCR with oligonucleotides 08.276 and 08.277, amplifying the wild-type (~440bp) and the GFP-fusion fragment (~2800bp). S: 2log-ladder; 2n: Exemplary PCR products from diploid transformant (KNH15). **C:** Haploid segregants were analyzed by fluorescence microscopy. The left panel displays the DIC-image, the right one detection through the EGFP-filter.

The complementation analyses described in section 3.2.2.3 for *KlINN1* suggested that the encoded protein cannot perform its function in a heterologous yeast host, and that this failure could be due to different membrane compositions in the two yeast species, which impede the proper interaction with the C2-domain of Inn1. Nevertheless, the essential nature of the encoding genes in both yeast species, and the fact that the *S. cerevisiae* homologue was shown to govern membrane ingression during cytokinesis, strongly indicates a similar function of *Kl*Inn1 in its natural host. In order to gain evidence for this hypothesis, a GFP-fusion of *Kl*Inn1 was obtained by recombination of the encoding sequences at the chromosomal *KlINN1* locus, similar to the strategies described above for *KlCYK3-GFP* and *KlHOF1-GFP.* Again, strain KHO70 was used as a recipient and after confirmation of the correct fusion event by PCR the strain was subjected to tetrad analysis (Fig. 3.27). The majority of seven tetrads investigated produced four viable colonies, suggesting that *Kl*Inn1-GFP functions properly *in vivo*. Fluorescence microscopy revealed GFP-signals at the bud neck of large budded cells, with a preference from the side of the daughter cell, where cytokinesis is presumably initiated.



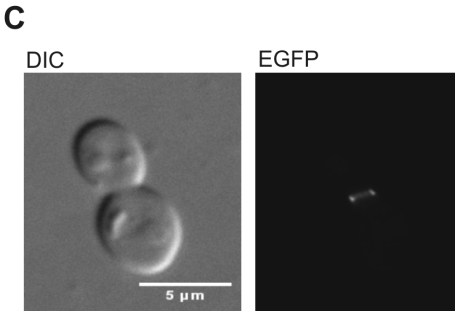
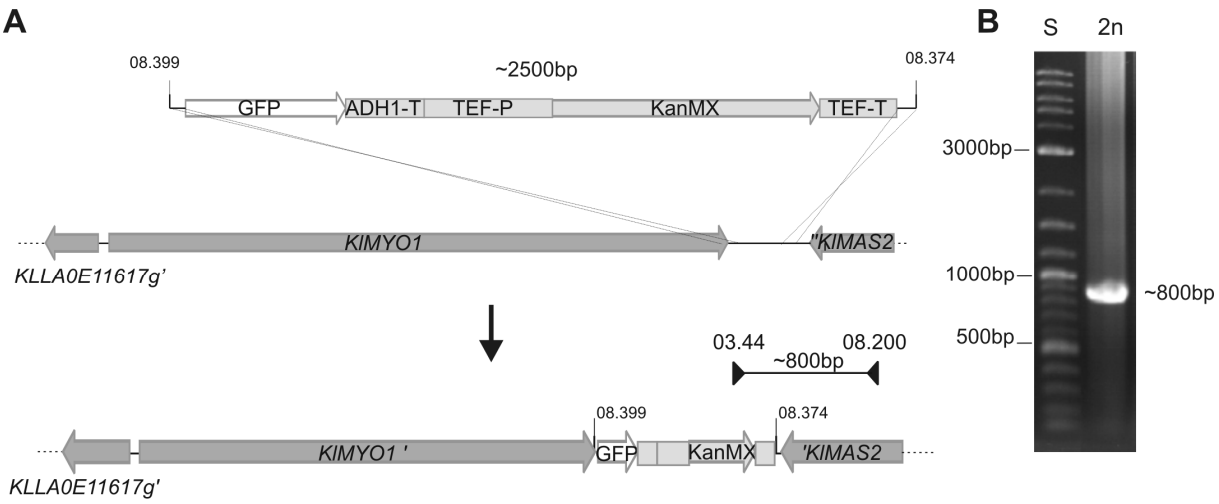


Fig. 3.27: *KlINN1*-GFP is localized at the bud neck.
A: The GFP-KanMX cassette was amplified from pFA6a-GFP-KanMX with oligonucleotides 08.175 and 08.83, resulting in a PCR product of ~2500bp. *K. lactis* diploid wild-type strain KHO60 was transformed with the cassette. **B:** Proper insertion of the GFP-tag was confirmed by PCR, using oligonucleotides 08.66 and 09.135, amplifying ~3500bp (GFP fusion) and ~1150bp (wild-type). S: 2log-ladder; 2n: exemplary PCR products from one diploid transformant (KNH20). **C:** Haploid segregants carrying the *KlINN1*-GFP fusion were subjected to fluorescence microscopy. The left panel displays the DIC-image, the right one detection through the EGFP-filter.

On average, these signals lasted for approximately 10 min, similar to the data reported for its homologue in *S. cerevisiae* (Jendretzki et al., 2009). These observations further support the view of *Kl*Inn1 functioning in yeast cytokinesis, but missing its interaction partners (e.g. membrane lipids) upon heterologous gene expression in *S. cerevisiae*.

In order to allow a more detailed timing of the cytokinesis events in *K. lactis,* the *KlMYO1* gene was also tagged at its 3'-end at its chromosomal locus with sequences encoding fluorescence markers, i.e. either GFP or mCherry (Fig. 3.28). In addition, plasmid-encoded copies of these alleles were obtained, in order to allow a fast introduction of the reporters into any *K. lactis* strain of interest. *Kl*Myo1-GFP encoded by the chromosomal allele was detected at the bud neck of *K. lactis*, similar to its *Sc*Myo1 homologue in bakers yeast. A preliminary kinetics of this signal was obtained by performing a time lapse analysis.



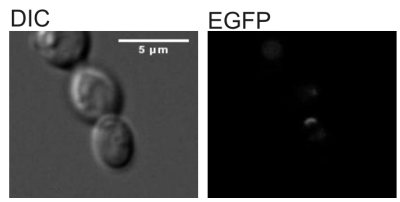


Fig. 3.28: *Kl*Myo1-GFP localizes to the bud neck.
A: The GFP-KanMX-fusion cassette was amplified from template pFA6a-GFP-KanMX by using oligonucleotides 08.399 and 08.374. Wild-type strain KHO70 was transformed with the PCR product (~2500bp). **B:** G418-resistant transformants were tested by PCR with oligonucleotides 03.44 and 08.200, amplifying the 3`-end of the GFP-fusion (~800bp). S: 2log-ladder; 2n: Exemplary PCR product from diploid transformant (KNH21). **C:** Haploid segregants were analyzed by fluorescence microscopy. The left panel displays the DIC-image, the right one the image through the EGFP-filter.

For this purpose, growing cells were monitored for eight hours (which corresponded to two cell divisions) under the microscope, and pictures were taken every three minutes. The *Kl*Myo1-GFP signal in a sample cell investigated was detected for 27 min at the bud neck, first appearing with a weak intensity when the daughter cell reached approximately half the size of the mother cell. During bud growth the signal intensified, with a maximum reached after 24 min. Within the following 10 min, fluorescence at the bud neck disappeared, coinciding with the separation of the daughter cell (Fig. 3.29).

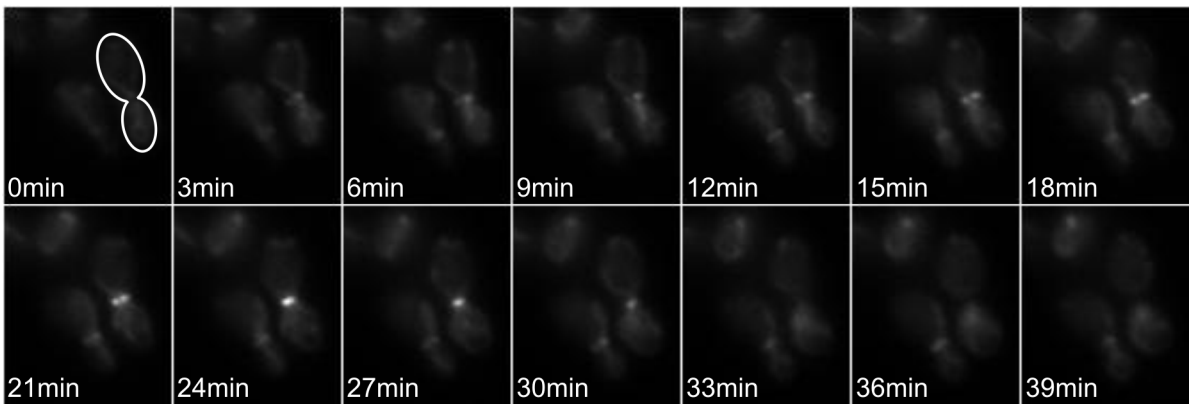


Fig. 3.29: *Kl*Myo1-GFP locates to the bud neck for ~24min during cytokinesis.
Cells expressing *KlMYO1*-GFP (KNH21) were subjected to time-lapse microscopy (section 2.2.10.3) at 30℃ in SC-medium. Every 3min EGFP-images were taken for 8 hours. The cell shape is outlined in white in the first image (0min).

A similar signal for the plasmid encoded *Kl*Myo1-mCherry fusion was also observed at the *K. lactis* bud neck (Fig. 3.30), whereas the chromosomal copy of this construct generally produced very weak signals and would need further optimization (data not shown). Nevertheless, these constructs will serve as valuable tools for future investigations of cytokinesis in *K. lactis.*

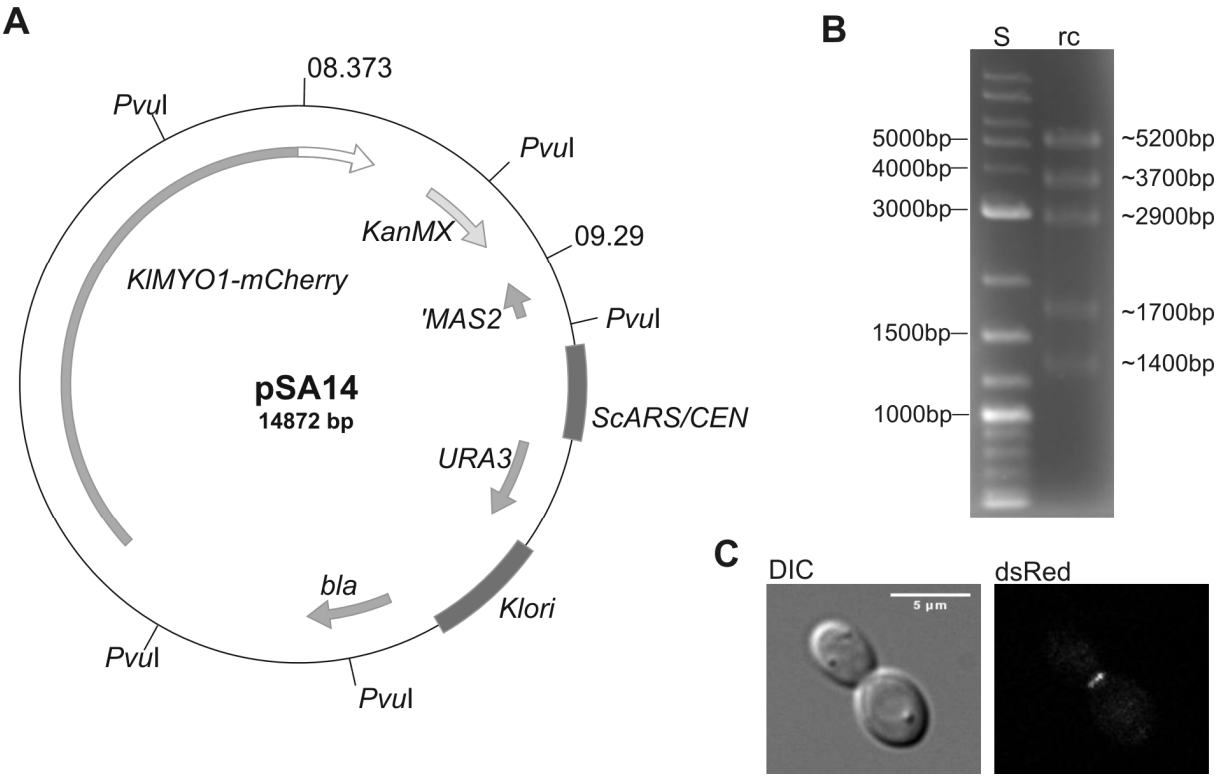


Fig. 3.30: *Kl*Myo1-mCherry localizes to the bud neck.
A: Plasmid pSA14 is comprised of the *KlMYO1*-mCherry fusion and the backbone of vector pCXS22. For construction, oligonucleotides 08.373 and 09.20 and plasmid YCplac33-*KlMYO1* were employed (see Tab. 2.2 for cloning strategy). **B:** As restriction control, pSA14 was digested with restriction enzyme *Pvu*I (see A), resulting in five fragments of expected lengths. S: 2log-ladder; rc: restriction control. **C:** *K. lactis* strain KHO60 was transformed with pSA14 and transformants were subjected to fluorescence microscopy. The left panel displays the DIC-image, the right one the image through the dsRed-filter.

3.2.4 Yeast two-hybrid assays

The results presented so far demonstrated that in *K. lactis* the homologues of *Sc*Inn1, *Sc*Hof1 and *Sc*Cyk3 localize to the bud neck and that all of these proteins are essential. Moreover, morphological analyses of non-viable segregants indicated that, like their counterparts in *S. cerevisiae,* these proteins are also regulating cytokinesis in *K. lactis*. Since *Sc*Cyk3 and *Sc*Hof1 have been shown to physically interact with *Sc*Inn1 in *S. cerevisiae,* yeast two-hybrid analyses were performed with the *K. lactis* homologues to investigate, whether they exert similar interactions. The results are summarized in Fig. 3.31. Clearly, interactions were only observed between the two-

hybrid fusions derived from the *S. cerevisiae* proteins, as reported in the literature (i.e. *Sc*Cyk3-*Sc*Inn1, *Sc*Hof1-*Sc*Inn1; Jendretzki et al., 2009), but not between the corresponding pairs of the *K. lactis* constructs. Regarding the interspecies assays, only *Sc*Hof1 seems to interact with *Kl*Inn1, indicating that the PRM motifs of the latter are indeed recognized by the SH3-domain of the former. The apparent lack of other expected interactions is quite puzzling and would need further investigations, which due to time constraints could not be performed in the framework of this thesis.

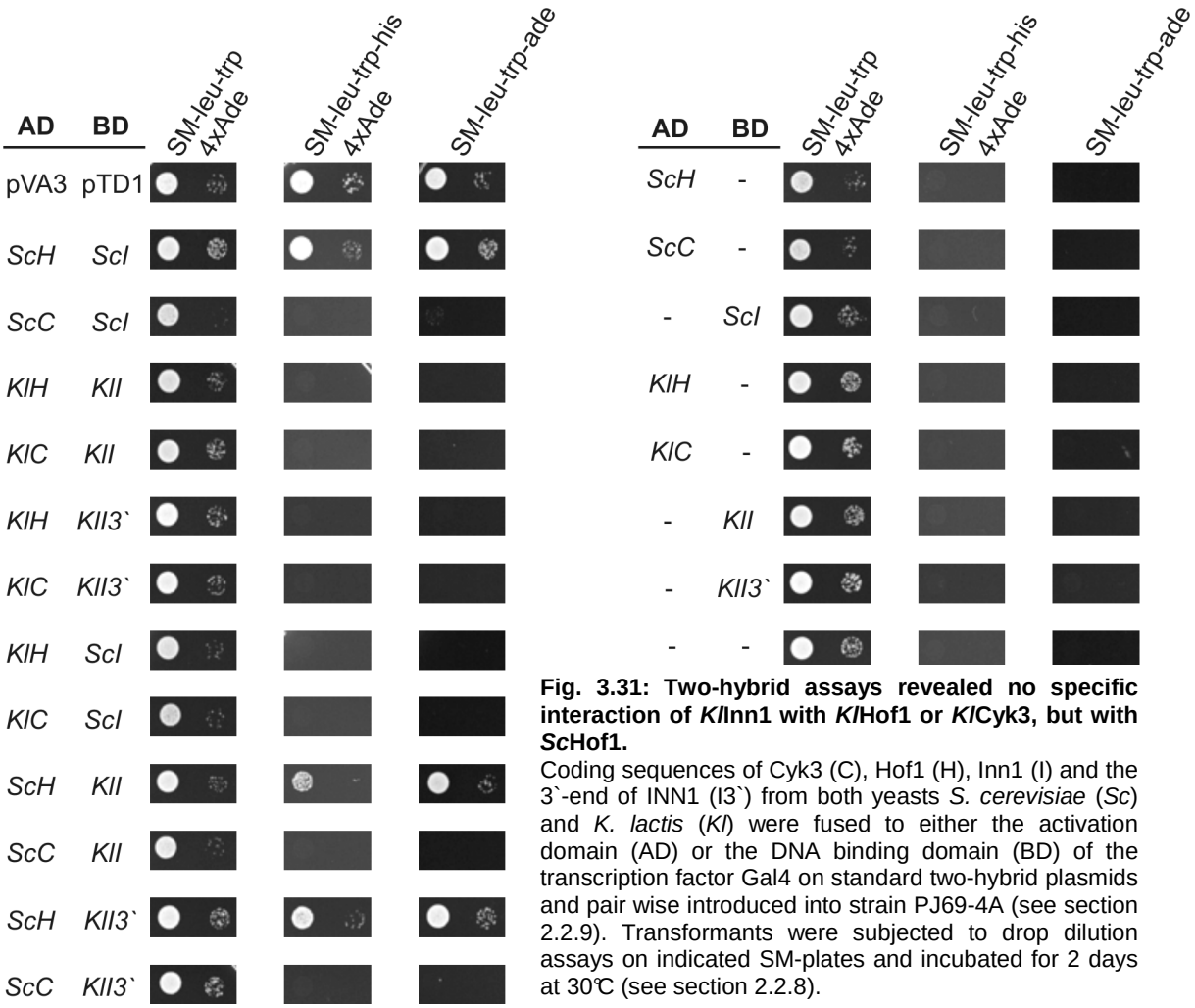


Fig. 3.31: Two-hybrid assays revealed no specific interaction of *Kl*Inn1 with *Kl*Hof1 or *Kl*Cyk3, but with *Sc*Hof1.
Coding sequences of Cyk3 (C), Hof1 (H), Inn1 (I) and the 3`-end of INN1 (I3`) from both yeasts *S. cerevisiae* (*Sc*) and *K. lactis* (*Kl*) were fused to either the activation domain (AD) or the DNA binding domain (BD) of the transcription factor Gal4 on standard two-hybrid plasmids and pair wise introduced into strain PJ69-4A (see section 2.2.9). Transformants were subjected to drop dilution assays on indicated SM-plates and incubated for 2 days at 30℃ (see section 2.2.8).

3.3 Analyses of budding patterns in *K. lactis*

Cytokinesis in *S. cerevisiae* is a highly-regulated event, which displays a distinct polarity. Thus, haploid strains follow an axial budding pattern, meaning that a new bud develops at the same pole of the mother cell, where the previous daughter cell emerged. In contrast, new buds in diploid strains generally develop at the pole opposite to the previous bud site or the birth scar of the new daughter cell (bipolar

budding pattern; reviewed in Casamayor and Snyder, 2002). The budding pattern in *K. lactis* has not yet been investigated with regard to this polarity. Thus, time-lapse DIC microscopy was performed on the haploid type strain CBS2359 and a diploid derivative from a congenic series (Fig. 3.32; Heinisch et al., 2010). During the cell divisions followed in this approach, both haploid and diploid strains displayed an axial budding pattern, with new buds emerging predominantly at the same pole of the cell where the previous budding event took place. In contrast, and similar to the observations in *S. cerevisiae,* the first bud of a daughter cell exclusively emerged at the pole opposite to the birth scar, i.e. in a bipolar pattern, in both haploid and diploid *K. lactis* strains. To substantiate these observations, budding patterns were quantified and results are shown in Table 3.2.

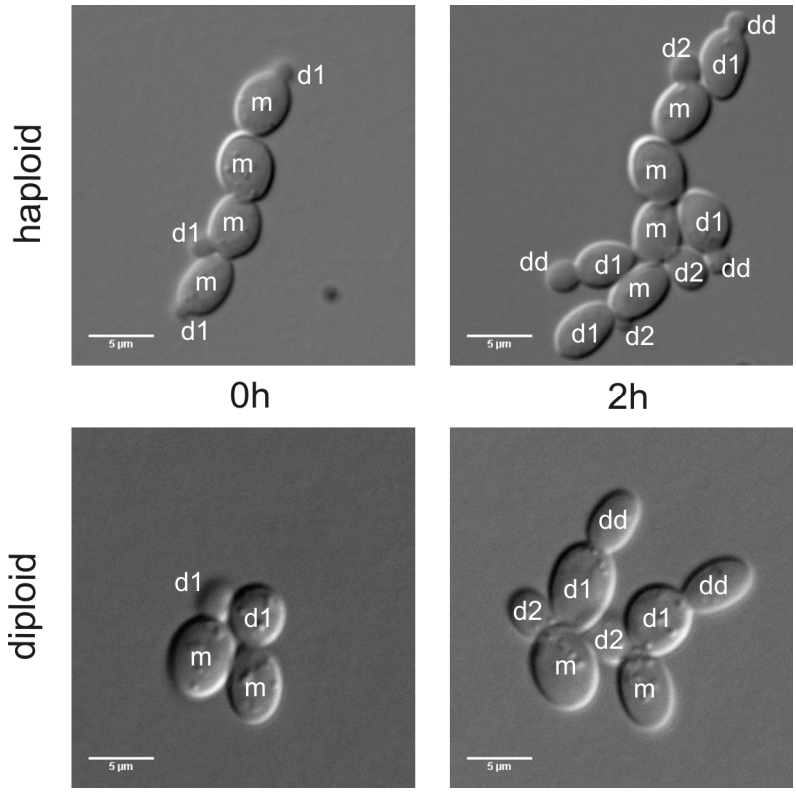


Fig. 3.32: Haploid and diploid K. lactis cells display an axial budding pattern.
Haploid strain CBS2359 (upper row) and a diploid derivative (lower row) were subjected to time-lapse microscopy (see section 2.2.10.3) at 30℃ on YEPD. For 3 hours DIC-images were taken every 7min. Exemplary pictures show cells at t=0min (left hand) and 2 hours later (right hand). m: mother cell; d1: first daughter of mother cell; d2: second daughter of same mother cell; dd: daughter of first daughter cell.

Tab. 3.2: Budding patterns of *K. lactis* wild-type strains were quantified.

	axial	random	bipolar	total
CBS2359 (haploid)	36	1	5	42
KHO60 (diploid)	56	2	4	62

4 Discussions

The two lines of research followed in this thesis, i.e. the investigation of putative negative regulators of the CWI pathway in *S. cerevisiae*, and the studies on cytokinesis in *K. lactis*, will be discussed separately in the following.

4.1 Putative negative regulators of the CWI pathway in yeast

In a genetic screen intended to identify putative negative regulators of the CWI pathway, several hits in different genes were obtained (PhD thesis of H.-P. Schmitz; and unpublished data from this laboratory generated in the diploma thesis of Sascha Jakob). In the course of this thesis, three candidates, namely Nta1, Fig4 and Set4, were investigated in some further detail. To confirm the validity of the screen, strains harboring the original reporter construct (stably integrated at the *LEU2*-locus in a tandem copy, strain HAS107L) in conjunction with complete deletions of the *NTA1*, *FIG4* and *SET4* open reading frames, were assayed for their specific β-galactosidase activities. Whereas a *FIG4* wild-type strain carrying the same reporter construct showed the expected increase in pathway activity upon heat stress (as deduced from the indirect reporter assay), a *fig4* deletion led to an even stronger induction. Fig4 thus could be a negative regulator of the CWI pathway. However, one should keep in mind that any protein involved in any step of cell wall biosynthesis (e.g. governing the secretory pathway of cell wall proteins or carbohydrates), would cause cell wall stress upon its deficiency and thus lead to similar phenotypes. The increase in reporter enzyme activity could thus be a rather indirect effect. Yet, in that case one would also expect to have a higher basal activity under normal growth conditions, which is not the case for the *fig4* deletion. On the other hand, in comparison to the wild type, the *nta1Δ* integrant showed an increased activity at both tested conditions (30℃ and 37℃), and would thus hint to a general function of Nta1 in cell wall synthesis.

Unexpectedly, the *set4Δ* mutant did not display elevated β-galactosidase activities compared to the wild-type strain under any growth conditions. In fact, a somewhat lower activity was found. It should be noted, that a similar behavior was observed for other candidate genes originally isolated in the genetic screen for their increased reporter activities (as judged from the blue color on X-Gal plates). Re-introducing the reporter construct or even determining pathway activity by the degree of MAPK phosphorylation in Western blots frequently did not confirm the original data (J. J.

Heinisch, personal communication). This indicates that pathway induction first needs to be verified in future experiments with candidate genes prior to their further investigation. Nevertheless, in one case investigated in this laboratory, namely for the *INN1* gene (A. Jendretzki; see section on cytokinesis for further details), the induction of the CWI pathway would certainly be expected, but again cannot be verified in these types of assays. Consequently, as β-galactosidase reporter assays described before (Straede et al., 2007), at least regarding the yeast CWI pathway, seem to be subject to considerable variations and should be interpreted with caution.

Moreover, the first genetic screen was based on a transposon-insertion mutagenesis (Burns et al., 1994) and thus did not result in complete gene deletions. In fact, the Fig4 protein encoded by the *tn7* mutant is only truncated in its C-terminal 75 amino acids. In contrast, the insertion in the *NTA1* locus (*tn9* mutant) allows only for the production of an Nta1 peptide with the N-terminal 75 amino acids and thus is not likely to produce a functional protein. On the other hand, the transposon insertion in the third mutant (*tn4*) took place in the promoter region of the *SET4* gene and thus may at best influence the genes expression (PhD thesis of H.-P. Schmitz). Albeit these differences of the originally isolated mutants to the complete deletions of the protein coding sequences could explain phenotypic variations, none of the three deletions displayed any CWI stress-related phenotypes in drop-dilution assays. This again indicates that a direct connection of the encoded protein to CWI signaling is rather unlikely. However, none of the deletion mutants displayed an increased resistance to cell wall stressing agents, as it would be expected for cells lacking a negative regulator of the CWI pathway.

What then could cause an indirect effect on CWI signaling? – Although information on the three genes at hand in the literature and the data bases are scarce, they suggest some possible connections.

4.1.1 Role of Nta1

Nta1 has been described as an amidase, which removes the amide group from proteins carrying an N-terminal asparagine or glutamine (Baker and Varshavsky, 1995). An analysis of the known components of the CWI pathway then revealed, that only Rom1 could be a putative substrate for Nta1, since it starts with an N-terminal asparagine. One would than expect the two proteins to interact with each other. The lack of a yeast two-hybrid interaction between Nta1 and Rom1 observed in this work

could thus be interpreted as Rom1 not being a natural substrate for Nta1. Alternatively, the fusion proteins with the Gal4 protein domains may not be fully functional or the interaction may be too transient to produce a notable signal in this assay. Consistent with these observations, an HA-tagged version of Nta1 did not pull down any significant amount of Rom1 or any other proteins from yeast crude extracts (data not shown). An exchange of the N-terminal amino acid of Rom1 to serine by *in vitro* mutagenesis did also not lead to any noticeable change in the protein function tested in a *rom1 rom2* double deletion background (data not shown). Since Rom1 also seems to be the less important player in the redundant pair of these GEFs in the yeast CWI pathway (Ozaki et al., 1996), and no other signaling component could serve as a substrate for Nta1, this line of research was discontinued. However, an interaction of Nta1 with Mkk1, the MAP kinase kinase of the CWI pathway, was revealed recently using high-throughput microarrays (Fasolo et al., 2011). Since Mkk1 does not contain an N-terminal asparagine or glutamine residue as a substrate for Nta1, but rather an alanine, this interaction is most likely not based on the known enzymatic activity of Nta1. If it is an artifact frequently observed in high-throughput screens, or if Nta1 serve a yet unknown second function related to Mkk1, remains to be elucidated. It should be noted, that several attempts to uncover the role of Nta1 in any process related to CWI signaling, failed in the course of this thesis and therefore have not been included in the results section. These experiments comprise a comparison of the actin cytoskeleton between wild-type and *nta1* deletion strains, as well as the localization of a Nta1-GFP fusion protein to mitochondria (shown in a global analysis by Huh et al., 2003), or the sensitivity of the deletion mutant to oxidative stress.

4.1.2 Role of Fig4

FIG4 encodes a phosphatidylinositol 3,5-bisphosphate phosphatase (Rudge et al., 2004), which is involved in the regulation of membrane lipid composition. Therefore its deletion may alter the phosphoinositide levels within the plasma- and/or internal membranes, which in turn may well affect the trafficking or the function of the CWI sensors. In fact, the null mutant of *FIG4* has been reported to be hypersensitive to both heat and caffeine as compared to the wild type (de Groot et al., 2001; Dudley et al., 2005). This phenotype stands in contrast to what would be expected for a negative regulator of CWI signaling, but is consistent with the hypothesis of an

impaired sensor function. However, neither of these sensitivities could be reproduced in this thesis, possibly due to differences in the genetic backgrounds of the yeast strains employed (which was based on the Y800 strain series (Burns et al., 1994) in the work cited and on HD56-5A in this work). The use of Wsc1- and Mid2-GFP fusions to determine sensor localization in the wild-type and the *fig4* deletion strains also did not reveal any notable differences, indicating that intracellular trafficking of the sensors is not impaired. Epistasis analyses between a *fig4* deletion and either an *mpk1*- or a *wsc1 mid2* double deletion first showed a suppression of the CWI phenotypes by the *fig4* defect in drop dilution assays under various stress conditions (e.g. resistance to caffeine and Congo Red [see results section 3.1]). On the other hand, overexpression of *FIG4* from the strong *GAL1* promoter did not yield any marked phenotype. The latter observation could be explained by the fact, that Fig4 has been reported to form a complex with Vac14, which was not overproduced here (Dove et al., 2002; Gary et al., 2002; Rudge et al., 2004). It should be noted that after repeated incubation of the double and triple deletion mutants, the epistasis relations described above could not be reproduced in drop dilution assays, indicating that subtle changes in medium conditions may have a major effect on these tests.
Due to this high variability and the fact that neither the *nta1*, nor the *fig4* deletion mutants, showed the expected hyper-resistances to cell wall stress or gave any strong indication for a connection to CWI signaling, the entire line of research was discontinued.

4.2 A comparison of cytokinesis in *S. cerevisiae* and *K. lactis*

In the above mentioned screen for putative negative regulators of the CWI pathway in *S. cerevisiae*, one then uncharacterized ORF was found: *YNL152w*. It was shown, that the encoded protein, now named Inn1, is essential for ingression of the plasma membrane during cell division (Sanchez-Diaz et al., 2008), and thus performs a vital role in cytokinesis. The relations of Inn1 with two other key regulators for cytokinesis, Hof1 and Cyk3, have been investigated extensively (Sanchez-Diaz et al., 2008; Jendretzki et al., 2009; Nishihama et al., 2009.). The current model for Inn1 function in *S. cerevisiae* derived from these data is shown in Fig. 4.1. In brief, the proline-rich motifs in the C-terminal half of the protein interact with the SH3-domains of both Hof1 and Cyk3. The latter are recruited to the bud neck during cytokinesis. Inn1 is believed

to mediate the interaction with plasma membrane lipids via its C2-domain and thus couple septum formation with plasma membrane ingression.

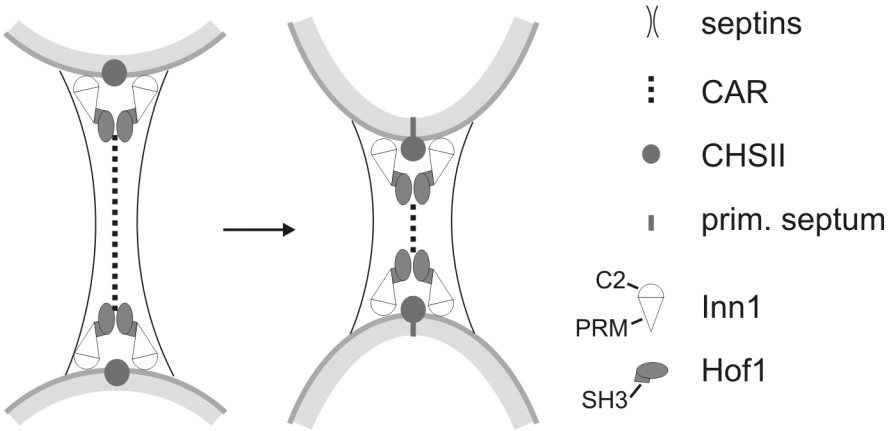


Fig. 4.1: Hypothetical model of how Inn1 functions during cytokinesis (modified from PhD thesis of A. Jendretzki)
Inn1 is recruited to the bud neck by interaction of its PRMs with the SH3-domain of Hof1. Via the C2-domain Inn1 binds to the plasma membrane and, thus, mediates its ingression during CAR constriction. In parallel, the primary septum is built into the cleavage furrow upon chitin deposition by chitin synthase II.

4.2.1 Budding pattern in K. lactis

Cytokinesis in *K. lactis* has not been studied in detail until now. Despite its similar way of reproduction by budding and the close evolutionary relationship to *S. cerevisiae,* this milk yeast has a fundamentally different metabolism, which primarily relies on energy production through the respiratory chain (Tarrio et al., 2006). A first hint to differences in its budding pattern was obtained in this thesis. *S. cerevisiae* shows distinct polar budding patterns depending on its ploidy. Thus, diploid cells form a new bud at the pole opposite to the one used for the previous bud formation, whereas haploid cells tend to form new buds at the same pole, where the previous daughter cell has separated (reviewed in Casamayor and Snyder, 2002). Here, no marked differences regarding the budding pattern could be observed between haploid and diploid strains of *K. lactis*: Both strains displayed an axial budding pattern. This indicates that no differential regulation in bud site selection occurs in this yeast. In order to designate homologues of the proteins involved in bud side selection in *S. cerevisiae* (i.e. Bud3, Bud4, Axl2, Axl1 for axial budding and Bud8, Bud9, Rax2 for bipolar budding) a genome wide BLAST search in *K. lactis* has been performed (Tab. 4.1). Apart from Axl1 and Bud9, to each protein could be assigned to a potential homologue with amino acid identities of the deduced proteins ranging from 34% to 57%. While *Sc*Axl1 seems to be encoded by two genes in

K. lactis, no homologue could be unambiguously assigned to *Sc*Bud9. Instead, the ORF which was referred to as homologue of *Sc*Bud8 was suggested again (Tab. 4.1, marked in gray). Interestingly, the protein with the two assigned homologues in *K. lactis*, Axl1, is involved in regulation of the axial budding pattern, which seems to be the dominant budding pattern in both haploid and diploid strains in milk yeast (see in results section Tab. 3.2), whereas the protein with no putative homologue, Bud9, mediates the bipolar budding pattern, which rarely occurs in *K. lactis.* Moreover, a *Scbud9* mutant displays an alteration of budding pattern from bipolar to distal (reviewed in Casamayor and Snyder, 2002), indicating that the predominant axial budding pattern in *K. lactis* may be due to the lack of a Bud9 homologue. Clearly, heterologous gene expressions in both yeasts will be necessary to determine if any of the putative homologues are really functionally equivalent.

Tab. 4.1: Proteins involved in bud site selection in *S. cerevisiae* and assigned homologues in *K. lactis*

	Budding pattern in *S. c.*			
	in wild-type	in mutants	**Homologue in *K. l.***	**AA Identity**
*Sc*Bud3	axial	bipolar	KLLA0D04994g	39%
*Sc*Bud4	axial	bipolar	KLLA0E06007g	40%
*Sc*Axl1	axial	bipolar	KLLA0D15361g	37%
			KLLA0E05105g	35%
*Sc*Axl2	axial	bipolar	KLLA0C11407g	57%
*Sc*Bud8	bipolar	proximal	KLLA0A02541g	41%
*Sc*Bud9	bipolar	distal	KLLA0A02541g	43%
*Sc*Rax2	bipolar	axial-like	KLLA0F18975g	34%

4.2.2 The cytokinesis regulators *Kl*Inn1, *Kl*Hof1, and *Kl*Cyk3

Based on these differences in the determination of the bud site, and in view of the main interest of the laboratory in the regulation of cytokinesis in *S. cerevisiae* as described above, homologues of three of the key regulators were investigated in the following in *K. lactis*. Thus, in addition to *KlINN1, KlHOF1* and *KlCYK3* were shown to be essential genes in this yeast. The latter two genes encode proteins with largely redundant functions in *S. cerevisiae* and are therefore not essential by themselves, but deletions display a synthetic lethality (Korinek et al., 2000). The main biological function of both proteins has been proposed to be the interaction with the proline-rich motifs (PRMs) of Inn1 through their respective SH3-domains (Jendretzki et al., 2009;

Nishihama et al., 2008). Mutants lacking the SH3-domain of either *Sc*Hof1 or *Sc*Cyk3 did not display any growth defects, presumably because Inn1 is still recruited to the bud neck by the remaining functional protein, which mediates a proper cytokinesis. However, further investigations revealed functional differences between *Sc*Hof1 and *Sc*Cyk3, indicating that *Sc*Cyk3 mediates additional tasks. Whilst *Sc*Hof1 still localizes to the bud neck after removal of its SH3-domain (Lippincott and Li, 2000), proper localization of *Sc*Cyk3 to this microcompartment depends on its SH3-domain (Jendretzki et al., 2009). Moreover, it was shown that upon depletion of *Sc*Inn1, *Sc*Cyk3-GFP remains at the bud neck, although severe morphological defects are observed. It was concluded that bud-neck localization of *Sc*Cyk3 is independent of the presence of *Sc*Inn1. Amongst eukaryotes, *S. cerevisiae* is somewhat peculiar, since cytokinesis can be accomplished by two different pathways: the first is dependent on the contractile actomyosin ring (CAR), the second is independent of the CAR and requires *Sc*Cyk3, presumably to recruit *Sc*Inn1. Kinetics of the accumulation of the different regulators at the bud neck revealed that *Sc*Cyk3-GFP appears during CAR constriction and vanishes only after disassembly of the latter. In contrast, *Sc*Hof1-GFP first appears as a double ring-structure early in cytokinesis, which then fuses during CAR constriction and relocates to the septin collar when CAR constriction has completed (Lippincott and Li, 1998). In coincidence with the disassembly of *Sc*Cyk3-GFP, *Sc*Hof1-GFP disappears here, probably marking the time of completion of septum formation. Interestingly, *Sc*Chs2, the chitin synthase responsible for the formation of the primary septum, displays a similar kinetics at the bud neck as *Sc*Cyk3 (Roh et al., 2002) (pers. communication with A. Jendretzki). It has therefore been suggested, that in *S. cerevisiae* Cyk3 and Chs2 are not actually components of the CAR, but rather form a second ring-like structure, which may mediate cytokinesis in a CAR-independent manner.

In general, *Kl*Myo1, *Kl*Hof1, *Kl*Cyk3 and *Kl*Inn1 show a similar intracellular distribution in *K. lactis* as their homologues in *S. cerevisiae*. Thus, in a first approximation one may conclude that they serve similar functions in both yeasts. It should be noted that molecular manipulations, such as gene fusions to the GFP coding sequence at the original chromosomal locus, are much more difficult to obtain in *K. lactis* as compared to *S. cerevisiae.* Due to these obstacles, the relevant constructs could be obtained in this thesis, but not employed for further extensive analyses. One such important issue for future investigations would be co-localizations to determine the temporal

appearance of the respective components at the bud neck, as described above for *S. cerevisiae*. This is of special interest, since the lethality of either a *Klhof1*- or a *Klcyk3* deletion suggests, that the redundant functions of these components in *S. cerevisiae* are not conserved in *K. lactis.* Together with the fact, that a *Klmyo1* deletion displays no major cytokinesis defects (see results section 3.2.1.4), this suggests, that in *K. lactis* a CAR-independent pathway may be operating, in which *Kl*Hof1 and *Kl*Cyk3 serve successive, rather than redundant functions. Given the conservation of their SH3-domains and the presence of the *Kl*Inn1 homologue, it is tempting to speculate that these functions could be the recruitment of the latter to the bud neck. *Kl*Inn1 would then mediate the plasma membrane ingression, necessary to accomplish a proper cytokinesis. Clearly, further investigations, based on the strains and constructs provided here, will be necessary to support this hypothesis.

Although all of the GFP-fusions of homologues of *S. cerevisiae* proteins investigated here in *K. lactis* (*Kl*Inn1-GFP, *Kl*Hof1-GFP, *Kl*Cyk3-GFP, *Kl*Myo1-GFP) localize to the bud neck during cytokinesis, cross-species complementation analyses suggest, that they are involved in at least partially different regulatory networks in the two yeast species. Among them, Hof1 probably diverged the least in its biological function, since heterologous complementation was observed for both species (i.e. *ScHOF1* complements the defects of a *Klhof1* deletion and *vice versa*). Nevertheless, the lethality of the deletion in *K. lactis* suggests, that the *Kl*Hof1 protein serves a unique and essential function in its original host. This would be in line with the above hypothesis of a sequential action of *Kl*Hof1 and *Kl*Cyk3 in cytokinesis. The two proteins could serve to ensure the presence of *Kl*Inn1 at the bud neck for plasma membrane ingression and/or abscission in a CAR independent pathway. This view is somewhat contradicted by the observation, that neither *Kl*Hof1, nor *Kl*Cyk3 interact with *Kl*Inn1 in a yeast two-hybrid assay. In contrast, *Kl*Inn1 was shown to interact (most likely through its PRM motifs) with *Sc*Hof1 (see results section 3.2.4). One possible explanation would be the masking of the interaction regions in the two-hybrid constructs of *Kl*Hof1 and *Kl*Cyk3 by some secondary protein structure. Such an influence is not unprecedented. In fact, a difference of only a few amino acids in the two-hybrid construct of a Rom2 fusion (the major GEF in CWI signaling) drastically changes its behavior regarding the interaction with the cell wall sensor Wsc1 (unpublished results from this group). To evaluate this hypothesis, different

two-hybrid constructs with various linker regions to the Gal4 regulatory domains should be tested in the future.

Interestingly, a gene disruption in *Kl*HOF1, which leaves the N-terminal half of the encoded protein untouched, displayed strongly aberrant cell morphologies, with elongated cells that are reminiscent of cytokinesis defects in *S. cerevisiae* (see results section 3.2.1.2). In view of the lethality of a complete *Klhof1* deletion, this provides strong evidence that the protein is indeed a key regulator of cytokinesis also in *K. lactis.*

In contrast to Hof1, complementation of the lethal phenotype of a *Klcyk3* deletion by *ScCYK3* could not be shown here. Since plasmids apparently are distributed very poorly in meiosis in *K. lactis*, this could be explained by a lack of segregation, rather than a lack of complementation. Two other attempts of complementation analyses (i.e. mass spore analysis and chromosomal integration of the heterologous gene copy) also failed so far. Therefore, these rather time consuming analyses were postponed for the time of this thesis. Nevertheless, *KlCYK3* complements the lethality of a *Schof1ΔSH3 Sccyk3ΔSH3* double deletion strain, indicating that cross-species function is retained at least in one direction.

On the other hand, a clear species-specific function could be determined for the Inn1 protein. Thus, *ScINN1* failed to complement the lethality of a *Klinn1* deletion, as did *KlINN1* in the respective deletion strain of *S. cerevisiae*. Given the proposed structure and function of *Sc*Inn1, this could be explained either by a lack of proper interaction of the C2-domain with the heterologous membranes, or by a lack of interaction of the PRM motifs with the proper target proteins. Since at least *Kl*Inn1 interacts with *Sc*Hof1 in the yeast two-hybrid assay (see above), the latter possibility seems unlikely. Indeed, chimeric proteins between *Sc*Inn1 and *Kl*Inn1 demonstrated that the C2-domain probably provides the necessary specificity for each species (see results section 3.2.2.3). Unpublished data from this group indicate that the C2-domain of *Sc*Inn1 preferably interacts with phosphatidic acid in *S. cerevisiae* (A. Jendretzki, personal communication). One may then speculate that *Kl*Inn1 either interacts with a different membrane lipid, or that local membrane compositions at the bud neck differ significantly between the two yeasts. No literature data are available on this topic in *K. lactis.* However, it should be noted that other yeast species carrying an *INN1* homologue do not significantly depend on its function. Thus, a *fic1* deletion in *Schizosaccharomyces pombe* has no cytokinesis defect (Roberts-Galbraith et al.,

2009). In this yeast, which is only distantly related to *S. cerevisiae* and *K. lactis*, cytokinesis also does not involve a chitinous primary septum, indicating that the entire process may be differently regulated.

Finally, a *Klmyo1* deletion does not display any strong phenotypes when growing at 30℃ in rich medium. At elevated temperatures, elon gated cells with morphological defects can be found. The latter observation indicates a role for *Kl*Myo1 in cytokinesis, whereas the apparently normal cell division at lower temperatures shows that it is not as important as its homologue in *S. cerevisiae*. Nevertheless, it appears at the bud neck during cell division and is probably also a component of a CAR structure in *K. lactis.* Again, this hypothesis will need further detailed investigations, since no literature data are available on this ring structure in *K. lactis*. The fact that *KlMYO1* fails to complement the cytokinesis defects of a *Scmyo1* deletion indicates, that whatever purpose the encoded protein serves in *K. lactis* has allowed it to evolve sufficiently different functions.

In summary, the data provided on cytokinesis in *K. lactis* in this thesis may serve as a starting point for further detailed investigations. For example, the GFP- and mCherry fusions of *Kl*Myo1 could serve as a timer for actomyosin ring formation and constriction, similar to their use in *S. cerevisiae* (Jendretzki et al., 2009). The data obtained so far strongly indicate, that at least some of the regulatory components identified serve different functions in *K. lactis* as compared to their *S. cerevisiae* homologues. In combination with the less redundant genome and a metabolism more reminiscent of mammalian cells than that of *S. cerevisiae*, *K. lactis* can be expected to yield new and exciting results regarding the fine regulation of cytokinesis in fungi and higher eukaryotes.

5 Summary

Cell division and the maintenance of cellular integrity are key features of life itself and some vital aspects of these processes have been studied in this thesis, using the yeasts *Saccharomyces cerevisiae* and *Kluyveromyces lactis* as model eukaryotes.

In the first part of the thesis, three putative negative regulators of the cell wall integrity (CWI) signal transduction pathway were investigated, which have been isolated in previous genetic screens. Whereas the *FIG4* gene seems to encode a protein which could be distantly related to CWI signaling, *NTA1* and *SET4* gene products were not found to have a major influence, as judged from phenotypes of deletion mutants, overproducers, and epistasis analyses with different CWI pathway mutants. In general, the data indicated rather indirect connections of all three protein functions with the maintenance of cellular integrity. Therefore, this line of research was discontinued, in order to investigate more closely the regulation of cytokinesis in the dairy yeast *K. lactis*.

In this second and major part of the thesis the homologue of a recently found cytokinesis regulator in *S. cerevisiae* (*INN1*, accordingly designated as *KlINN1*), was cloned and characterized. It could be shown, that the gene is essential and that the encoded protein is species-specific, i.e. *KlINN1* does not complement the lethality of a *Scinn1* deletion mutant and *vice versa*. Analyses of hybrid proteins demonstrated that this specificity is most likely mediated by the C2-domain of the protein, which is thought to interact with membrane lipids. In *S. cerevisiae*, Inn1 interacts through its proline-rich motifs located in the C-terminal half of the protein with the cytokinesis regulators Hof1 and Cyk3. They both carry a SH3-domain which has been shown to mediate the interaction with Inn1. Consequently, the two encoding genes, *KlHOF1* and *KlCYK3*, were also characterized in *K. lactis*. In contrast to *S. cerevisiae*, where the homologues seem to exert somewhat redundant functions and only a double deletion is lethal, each of the genes is essential in *K. lactis*. The exact nature of their roles in cytokinesis of this yeast remains to be determined. Unexpectedly, attempts to confirm an interaction between the proline-rich motifs of *Kl*Inn1 and the SH3-domains of *Kl*Hof1 and *Kl*Cyk3 in a yeast two-hybrid assay failed so far, but this line of research will be followed up in future experiments.

In order to compare the timing of cytokinesis and the localization of the above mentioned regulators between *S. cerevisiae* and *K. lactis*, another homologue of a

protein involved in cytokinesis in *S. cerevisiae* was investigated in *K. lactis.* Preliminary evidence from deletion of the *KlMYO1* gene, which encodes a likely component of the contractile actomyosin ring (CAR) in *K. lactis*, indicates that this yeast may predominantly engage in a CAR-independent pathway of cytokinesis. Nevertheless, similar to *S. cerevisiae* GFP-fusions of *Kl*Inn1, *Kl*Hof1, *Kl*Cyk3, and *Kl*Myo1 all were shown here to localize to the bud neck during cytokinesis in *K. lactis*. In summary, components identified to play a crucial role in yeast cytokinesis in *S. cerevisiae* display a similar localization in *K. lactis*, but may differ considerably in their detailed functions *in vivo*. This thesis represents the first detailed investigation of the molecular processes underlying cytokinesis in *K. lactis* and provides the basis for elaborate future studies.

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7 List of abbreviations

AD	Activation domain
BD	DNA-Binding domain
CAR	Contractile actomyosin ring
CWI	Cell wall integrity
DIC	Differential interference contrast (microscopy)
DNA	Desoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
e.g.	For example (from latin *exempli gratia*)
G1	Gap one phase (cell cycle)
G2	Gap two phase (cell cycle)
G418	Geneticin
Gal	Galactose
GAP	GTPase activating protein
GDP	Guanosine diphosphate
GEF	Guanine nucleotide exchange factor
GFP	Green fluorescent protein
Glu	Glucose
G-protein	Guanine nucleotide binding protein
GTP	Guanosine triphosphate
i. e.	that is (from latin *id est*)
KAc	Potassium acetate
Lac	Lactose
M	Mitosis phase (cell cycle)
Mat	Mating type
OD_{600}	Optical density at a wavelength of 600 nm
PCR	Polymerase chain reaction
PEG	Polyethylene glycol
pH	negative logarithm (log_{10}) of the hydroxonium concentration
PRM	Proline rich motif
RNA	Ribonucleic acid
S	Synthesis phase (cell cycle)

SC	Synthetic complete medium
SDS	Sodium dodecyl sulfate
UV	Ultraviolet radiation
wt	Wild-type
X-Gal	5-Bromo-4-chloro-3-indolyl b-D-galactopyranoside
YEPD	Yeast extract peptone dextrose (rich medium)
YNB	Yeast nitrogen base

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

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9 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 uner Angabe der Quelle gekennzeichnet.
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> Ein Teil der hier präsentieren Ergebnisse wurden gemeinsam mit Studenten im Rahmen von Praktika und Abschlussarbeiten, die von mir während dieses Projektes betreut wurden, erarbeitet. Dabei sind die Abbildungen 3.13-3.16, 3.24, 3.28 und 3.29 in Zusammenarbeit mit Sabina Albermann entstanden.

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