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Role of Knr4 protein in *Saccharomyces cerevisiae* morphogenesis and sensitivity to Killer toxin K9: localization versus Phosphorylation

Ran Liu

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THÈSE

En vue de l'obtention du

DOCTORAT DE L'UNIVERSITÉ DE TOULOUSE

Délivré par :

Institut National des Sciences Appliquées de Toulouse (INSA de Toulouse)

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THESE

Présentée à

L'Institut National des Sciences Appliquées de Toulouse

par

Ran LIU

Le 4 mai 2015

en vue de l'obtention du

DOCTORAT

Sciences Ecologiques, Vétérinaires, Agronomiques et Bioingénierie,

Filière: Ingénieries microbienne et enzymatique

**Role of Knr4 protein in *Saccharomyces cerevisiae*
morphogenesis and sensitivity to Killer toxin K9:
localization *versus* Phosphorylation**

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May 2015

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Summary

KNR4 gene, whose name stands for “Killer Nine Resistant 4”, comes from its original isolation by a screen based on resistance to the Killer toxin K9 of *Hansenula mrakii* (Hong, Mann, Brown, et al. 1994a). Mutants defective in *KNR4* gene display reduced β -(1,3)-glucan synthase activity correlated with reduced levels of β -(1,3)-glucan in their cell walls (Hong, Mann, Shaw, et al. 1994a).

In the budding yeast *S. cerevisiae*, Knr4 protein localizes at the sites of polarized growth such as bud tips and shmoo tips (Martin-Yken et al. 2003; A. Dagkessamanskaia et al. 2010). Both N-terminal and the C-terminal domains of Knr4 protein are disordered, but only the N-terminus of Knr4 is required for the protein correct localization at the bud tip (A. Dagkessamanskaia et al. 2010). In most *S. cerevisiae* genetic backgrounds *KNR4* gene is not essential for growth under standard laboratory conditions, however its deletion leads to growth defects under numerous stress conditions such as elevated temperature or presence of SDS, caffeine, antifungals (ex.: caspofungin) or cell wall affecting drugs like Calcofluor White and Congo Red. This wide range of phenotypes corroborates the role of Knr4 protein in transcriptional control of gene expression (Martin-Yken et al. 2003).

Knr4 is a conserved yeast hub protein (Adilia Dagkessamanskaia, Durand, et al. 2010), known to genetically and physically interact with several distinct partners with diverse cellular functions (F. Basmaji et al. 2006) (Dagkessamanskaia et al. 2001; Martin-Yken et al. 2003) (and *Saccharomyces Genome Database*, <http://www.yeastgenome.org>). Through these interactions, Knr4 notably influences the transcriptional control of a large number of genes among which numerous cell wall synthesis genes, including all three chitin synthase genes present in *S. cerevisiae* genome (Martin et al. 1999).

Moreover, Knr4 participates in the transcriptional control of G1/S transition during cell cycle progression (A. Dagkessamanskaia et al. 2010). Finally, *KNR4* gene has homologs in the whole fungal genome, and functional and genomic data on homolog genes from *Candida albicans* (Nett et al. n.d.), *Neurospora crassa* (Verdin et al. 2009) and *Schizosaccharomyces pombe* (www.pombase.org) indicate that their products likely perform very similar cellular functions in these organisms.

The aim of my thesis was to study the function of Knr4 in the cell wall synthesis, morphogenesis, and related signaling pathways. The content of my thesis is mainly divided into three parts:

The first part concerns our search to find out unknown partners of Knr4 and to investigate the cellular pathways required for localization of Knr4 protein. To that end, we decided to use a series of deletion mutants interrupted in genes related to morphogenesis and establishment of cellular polarity. We selected candidate genes from the *Saccharomyces cerevisiae* genome database (SGD, Stanford), using the keywords “Morphogenesis” and “Cell Polarity”. After selection and addition, 25 genes related to the morphogenesis and cell polarity were chosen for our Knr4 localization analysis. Through analysis of the results, we got 10 interesting mutants related to morphogenesis and polarity in which knr4 protein localization was affected: *bem2Δ*, *pcl1Δ*, *pcl2Δ*, *rrd1Δ*, *spa2Δ*, *tpd3Δ*, *bem1Δ*, *bnl1Δ*, *yck1Δ* and *bud6Δ*, and two additional mutants *pph21Δ* related to the *tpd3Δ* and *cna1Δ* involved in the calcineurin pathway.

The second part deals with a mutational analysis of in vivo phosphorylated residues of Knr4 in the function and localization the protein, as well as in the modulation of calcineurin activity and CWI pathway. We found that S200S203 phosphorylation mutants cannot rescue viability of a double mutant *bck1Δknr4Δ*, while they can rescue *slt2Δknr4Δ*. In addition, S200S203 phosphorylation mutants behave as the absence of Knr4 towards suppression of lethality caused by an hyperactivated Mkk1 allele. Also we found that the *knr4Δ* with KNR4^{S200AS203A} mutant can results in hyperactivation of the Calcineurin pathway compared to control situation. So serin 200 and serin 203 may be involved in the cross-talking with the calcineurin pathway and CWI pathway.

The third part is the study of K9 killer toxin’s strong cytotoxic activity against sensitive yeast strains, including *Saccharomyces cerevisiae*. Treatment with this toxin results in the formation of pores at the surface of the cells, and more specifically at places where cell wall synthesis is the most active, namely at the tip of growing buds or mating projections. Yeast cells treated with K9 toxin then die by releasing cytoplasm and cellular materials from these pores. In the yeast *S. cerevisiae*, Knr4 protein localizes at the sites of polarized growth (bud tips, shmoo tips), which are also the sites where the toxin forms pores in the cell wall. Mutants defective in KNR4 gene are remarkably resistant to this toxin. In this study, we analyzed for the first time the biophysical effects of K9 on the yeast cell wall using Atomic

Force Microscopy (AFM), a cutting edge technology that allows measuring the nanomechanical properties of living yeast cells, and their alterations by various drugs. To this end, we measured the effects of K9 toxin on the nanomechanical properties of the cell wall of *S. cerevisiae* wild-type cells and mutants deleted for KNR4 gene, at the short (2 h) and long term (20 h). Our results reveal an important cell wall remodeling occurring in wild-type cells already after 2 hours and only visible in *knr4*Δ mutant after 20 hours of treatment. Moreover, we investigated the role of Knr4 protein in the cells sensitivity towards the toxin. We were able to show that the presence of the N-terminal domain of Knr4 protein, which is required for its correct cellular localization at the bud tip during cell cycle, is essential for the toxin K9 wild-type sensitivity. In addition, a series of deletion mutants from the YKO collection in which the Knr4 cellular localization is also lost display a reduced sensitivity to the K9 toxin. Taken together, these results shed light on the importance of the proper localization of Knr4 protein at sites of intensive cell wall growth for the wild-type cells sensitivity to K9 killer toxin.

Key words: Yeast *S. cerevisiae*, Knr4, CWI, Calcineurin pathway, Localization, phosphorylation, K9, AFM

Chapter1 Introduction

1.1 The yeast Cell wall

The yeasts, which live in the wild, are exposed to a huge range of changing conditions in environment. The yeast cell wall, even though it appears rigid, is a highly dynamic structure which faces and adapts to the environmental changes.

During its life, the yeast cell grows in size and buds several times. The yeast cell wall, which is required to maintain cell shape and integrity, has to cope with dramatic local modifications to ensure the formation of buds and the cell divisions. The rigid cell wall is remodeled to accommodate cell expansion during vegetative growth, as well as during mating pheromone induced and filamentation related morphological changes.

1.1.1 The composition of the Yeast cell wall

The major features of the *Saccharomyces cerevisiae* cell wall architecture are well known now. The cell wall represents 20% to 30% of the cell dry weight (Smits et al. 1999). The structure of the cell wall is layered, comprising an electron-transparent inner layer and an electron-dense outer layer. The electron-transparent inner layer, which is responsible for the mechanical strength and elasticity for the cell wall mainly, contains β -1, 3-glucan, β -1, 6-glucan branches and a small fraction of chitin (Figure1). The electron-dense outer layer is mainly composed of highly glycosylated mannoproteins (Osumi 1998; Smits et al. 1999). The outer layer not only can protect the cell from the cell wall degrading enzyme and is also important for the cell-cell recognition during sexual agglutination and biofilm formation. There are two classes of cell surface glycoproteins composing the outer layer. GPI (glycosylphosphatidylinositol) proteins, which are members of one class, are linked to β -1, 3-glucan indirectly through β -1, 6-glucan (Klis et al. 2002) in the cell wall (Levin 2005; Smits et al. 1999). And the pir proteins as the other class can be linked with β -1, 3-glucan-chitin lattice directly.

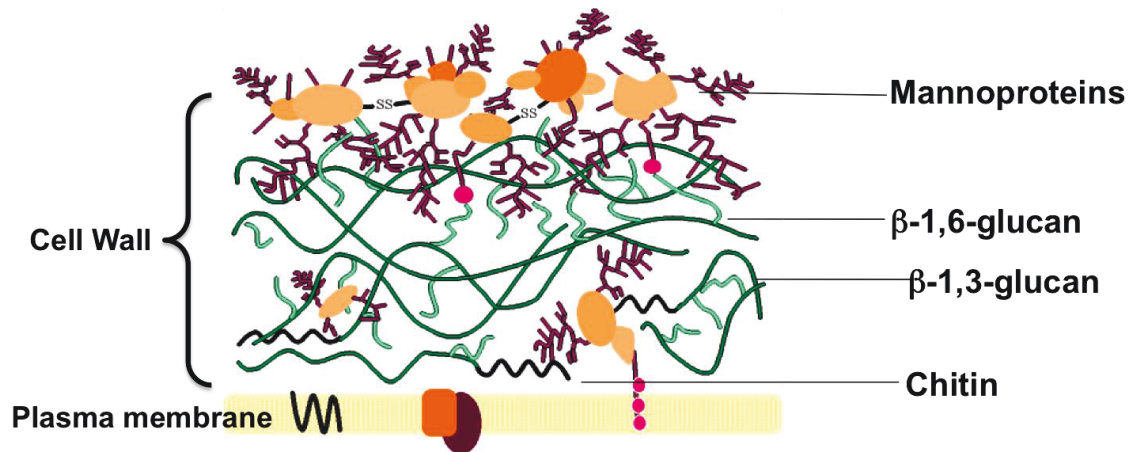


Figure 1: Cell wall structure

1.1.2 The yeast Cell wall as a target for Antifungal drug Development

Several fungi like *Candida* species can cause infections to humans as major opportunistic pathogens. Most of the currently used antifungal therapies are based on the azole class of molecules. They are inhibitors of ergosterol biosynthesis, and are cytostatic rather cytotoxic. It is thus still necessary to find cytotoxic antifungal drugs. Since the cell wall is a specific structure of fungi, absent in human cells, it is considered a great target for developing antifungal drugs. And *Saccharomyces cerevisiae* is a good model for the fungal cell wall biogenesis (Fostel & Lartey 2000; Gozalbo et al. 2004).

Caposfungin is a member of a new class of antifungals termed the echinocandins (Denning DW 2003). It inhibits β -1, 3-glucan synthase, the enzyme involved in synthesis of this major cell wall component. It has been recently used for clinic treatment of fungal infections, and the mechanism of the drugs is still not yet entirely clear, although *FKS1* gene mutations have been found in resistant isolates. Capofungin affects the cell division process by perturbing cytokinesis along with a diminution of β -1, 3-glucan content and an increase in chitin content.

1.1.3 The Cell wall integrity pathway – one of the pathways involved in the cell wall integrity

To maintain the cell wall integrity when yeasts meet stress environmental challenges, as well as during growth and morphogenesis, there are several pathways which interact with each other in *Saccharomyces cerevisiae*. The CWI (cell wall integrity) pathway is the principal pathway responding to these changes. CWI pathway, also called the PKC1 pathway, is a

linear cascade of Mitogen Activated Kinases (MAP Kinases) under the control of the budding yeast homolog of mammalian PKC protein. This pathway is regulated periodically through the cell cycle, peaking at the time of bud emergence, the time at which growth is most highly polarized (Zarzov et al., 1996).

1.1.3.1 The CWI MAP Kinase Cascade

Stress activation of this pathway occurs in response to a variety of external stimuli through a family of upstream membrane sensors (Wsc1, Wsc2, and Wsc3, Mid2 and Mtl1). These sensors are coupled to a small G protein called Rho1, functional homolog of the mammalian RhoA (Qatoda et al., 1994), which directly activates the yeast Pkc1 protein kinase. The cell integrity signalling from Pkc1 is then transmitted and amplified through a linear series of kinases: Bck1 (MAPKKK), Mkk1 and Mkk2 (a pair of redundant MAPKK), and the MAPK Slt2, final effector of the pathway and homolog of mammalian Erk5 (Figure 2). Activated Slt2 enters the nucleus, and activates transcription of cell wall related genes by interacting with at least two transcription factors: Rlm1 (which mostly controls cell wall genes expression) and SBF (involved in cell cycle G1 to S transition). The protein kinase activation domains of Erk5 and Slt2 share a very strong sequence identity, and it has been shown that expression of human Erk5 is able to replace Slt2 in *S. cerevisiae slt2Δ* mutants and provides cell integrity MAP kinase function in yeast (Truman et al., 2006). Analysis of human tumours has revealed a link between abnormal levels of ERK5 expression and cancer, leading to the suspicion that Erk5 signalling may be important for mediating the effects of several oncogenes (Hayashi and Lee, 2004, Wang et al., 2005). *KNR4* (see Introduction 1.4) is a member of the PKC1 pathway which genetically interacts with *BCK2* (Martin-Yken., 2002). A physical interaction between Knr4 and Slt2 was shown and proposed to modulate activation of transcriptional factors Rlm1 and SBF (Martin-Yken.,2002) by the MAP kinase. Knr4 is absolutely necessary for cell viability in the absence of a functional Pkc1-dependent cell integrity pathway (Durand et al, 2008).

1.1.3.1.1 The cell wall sensors

Five cell wall sensors (Wsc1, Wsc2, Wsc3, Mid2 and Mtl1) are implicated in transmitting the cell wall stress to GTPase Rho1 (Figure 2). These five sensors are transmembrane proteins whose structures are similar. And the sequences of Wsc proteins are similar to one another. Mid2 and Mtl1 share half sequence identity with each other (Levin 2005). Among these cell

wall stress sensors, Wsc1 and Mid2 appear to play a more important biological role than the others. A double mutant $\Delta wsc1\Delta mid2$ cannot survive without osmotic support (Rajavel et al. 1999). *WSC1* and *WSC2* isolated as dosage suppressors of the heat shock sensitivity of a hyperactive Ras/cyclic AMP pathway mutant (Verna et al. 1997). Only *WSC1* is isolated as a suppressor of the temperature-sensitive *swi4 Δ* , which is a component of the SBF transcriptional factor. Mid2 protein is important for the survival in the presence of mating factor, and is also required for Mpk1 activation in response to calcofluor white (Ketela et al. 1999; Philip & Levin 2001). The cytoplasmic domain of Wsc1 is phosphorylated and possesses two short regions for the interaction with Rom2. Phosphorylation of precise sites located within these interaction regions inhibits Wsc1 function. The localization of Wsc1 at the bud tip is dependent on the actin cytoskeleton, however the actin polarization is controlled by the Wsc1 (Delley & Hall 1999).

The cytoplasmic domain of Mid2 can interact with is a small plasma membrane-associated protein Zeo1. The phosphorylation site mutant is important for activation by wall stress (Vay et al. 2004). The mannosylation of the periplasmic ectodomains of Mid2 and Wsc1 is important to the function. Overexpressed Wsc1, but not Mid2, suppresses the GS activity defects of *fks1fks2* mutant (Sekiya-Kawasaki et al. 2002). It suggests that Mid2 activates Rho1, which is dedicated to the activation of Pkc1, but Wsc1 can be related to the activation of Rho1 effectors as well. Thus, Wsc1 and Mid2 play a partially overlapping role in CWI signalling. The product of *MLT1* gene appears also involved in the CWI signalling since this gene was identified as a dosage suppressor of a temperature- sensitive GS mutant, *fks 1-1154 fks2 Δ* . At last, all the sensors converge on activation of the Rho1 GTPase (Lommel et al. 2004; Philip & Levin 2001; Levin 2005).

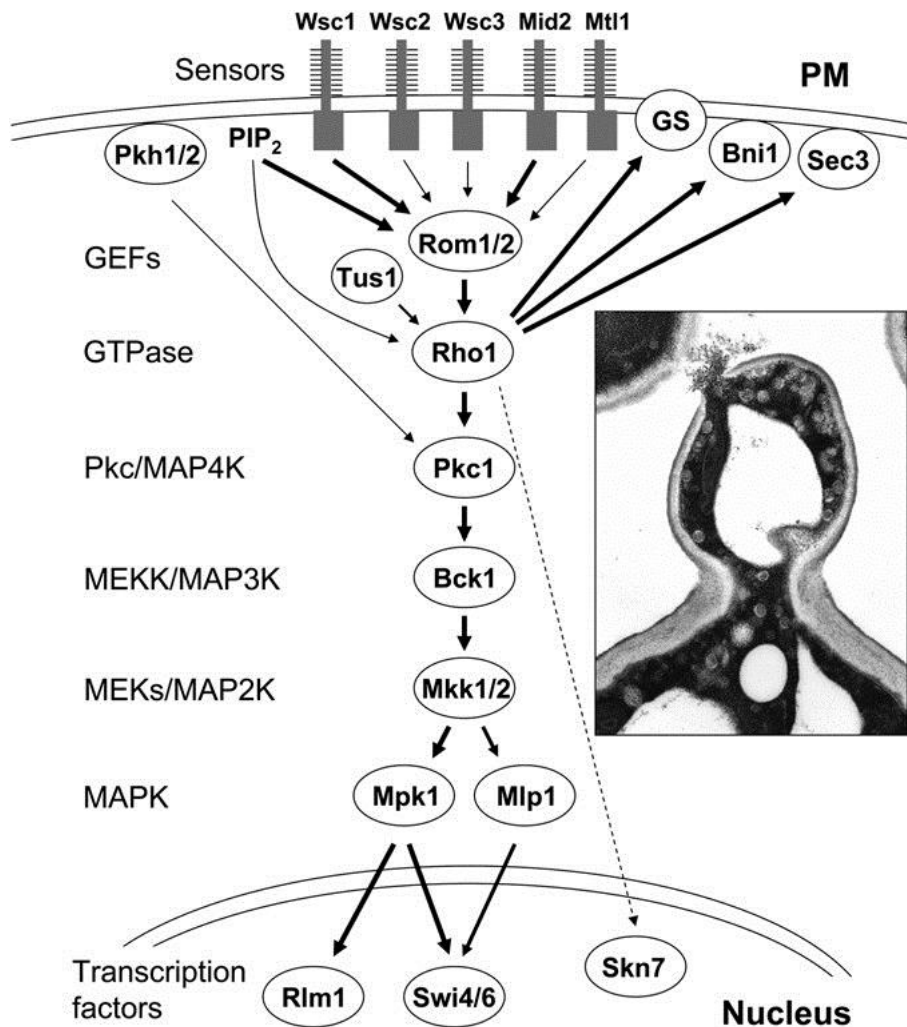


Figure 2: CWI signaling pathway. (Levin 2011)

1.1.3.1.2 The Rho1 GTPase

Rho1 protein is one of six members of the RHO GTPase family (Rho1, Rho2, Rho3, Rho4, Rho5 and Cdc42), which are involved in the control of polarized growth in *Saccharomyces cerevisiae*. Among these six members, Rho1 is important for the CWI signalling related to the activation of Pkc1, GS activity, the cytoskeleton organization and polarized secretion. Rho2 is redundant to Rho1. Rho3, which shares a role with Rho4 for the actin polarization and bud formation, is important for the cell growth. Rho5 can down regulate the CWI pathway (Levin 2005). Cdc42 is important for polarized growth and bud site assembly (Johnson 1999).

Rho1 protein, which is the master regulator of CWI pathway, is localized at the active sites of polarized growth. It is a GTP dependent protein, which is active in the GTP-bound state and inactive in the GDP-bound state. The Rho1 cycling between GTP-bound and GDP-bound is dependant on GTPase-activating proteins (GAPs) and guanosine nucleotide exchange factors

(GEFs). There are 11 Rho1-GAPs in the yeast *Saccharomyces cerevisiae* (Levin 2005). Rho1 is activated by Rom1 and Rom2 GEFs, whose deletions are synthetic lethal. Rom1 and Rom2 can interact with the GDP-bound Rho1 and possess the nucleotide exchange activity depending on the DH (Dbl Homology) domains. In addition, they bind the (Ip4, 5P₂) phosphatidylinositol-4, 5-biphosphate and are responsible for the proper localization of Rom1/2 in the plasma membrane through the PH domains (pleckstrin homology)(Audhya & Emr 2002). Tus1, another Rho1GEF, possesses both a DH domain and a PH domain and is needed in CWI pathway (Philip & Levin 2001).

There are 5 effectors downstream Rho1: the Pkc1 protein, the glucan synthase complex (GS), the two formins Bni1 and Bnr1, and the transcription factor Skn7. Altogether, these effectors regulate the cell wall synthesis and the polarization of the actin cytoskeleton (Levin 2005).

Among these 5 effectors, GS is the enzyme that catalyzes the synthesis of β -1, 3-glucan chains. There are three identified components of the GS complex (Klis et al. 2002). Fks1 and Fks2 are the catalytic subunits of the GS. Fks1 and Fks2 are multispinning integral membrane proteins, either one of them is enough for GS activity and cell viability. FKS3, another homolog of FKS1 and 2, is present in *S. cerevisiae* genome, but its product has not been characterized yet. And Rho1 is the regulatory subunit of the complex that stimulates GS activity in a GTP-dependent manner. Both Fks1 and Rho1 localize to the plasma membrane at the bud tip of the budding cell and at the bud neck during cytokinesis. *FKS1* is the gene-expressed predominantly in normal growth conditions. *FKS1* expression is under the control of transcriptional factors SBF and MBF and is weakly regulated by CWI pathway (Inoue et al. 1995; Lagorce et al. 2003)(Lagorce et al. 2003; Inoue et al. 1995). Inversely, *FKS2* seldom expresses under the optimal growth conditions but it can be induced upon treatment by mating pheromone, high temperature, growth on poor carbon sources, entry into stationary phase or in the absence of *FKS1* function (Levin 2005).

The CWI pathway and Calcineurin pathway are synthetically lethal. Overexpression of Mkk1 or Rlm1 can suppress the synthetic lethality of a $\Delta fks1 \Delta cnb1$ mutant. And expression of calcineurin suppresses the growth defects of $\Delta pkc1$ and $\Delta mpk1$. The expression of *FKS2* is controlled both the by calcineurin and CWI pathway through separable promoter elements. *FKS2* can be induced by Crz1 which binds to calcineurin-dependent response element (CDRE) in response to the high temperature (Zhao et al. 1998; Stathopoulos & Cyert 1997),

and expression of *FKS2* is also controlled by Rlm1 under the chronic stress and largely regulated by Swi4 in response to all stress. It thus appears that Rho1 can control the activity of the GS both during normal growth conditions and under cell wall stress (Levin 2005).

Remarkably, GS complex is the target of the echinocandin antifungal agents which affect directly the production of β -1, 3-glucan (Wigge et al. 1998).

Bni1 and Bnr1 are formins, responsible of the nucleation and assembly of linear actin filaments. They are components of the polarisome that reside at the cell cortex during bud growth.

Skn7 is one of two response regulators (Skn7, Ssk1) of the HOG (High osmolarity glycerol) pathway. Skn7 is regulated not only by the turgor sensor Sln1 in HOG pathway but also by the Rho1 in CWI pathway, showing some cross talk between these two MAP kinases cascades. However, Ssk1 is only regulated by Sln1 (Levin 2005).

1.1.3.1.3 Pkc1 protein Kinase

Pkc1 protein is the single homolog of mammalian protein kinase C in the yeast *Saccharomyces cerevisiae* and regulates the Bck1 MAP kinase cascade (Levin et al. 1990). *PKC1* is the first component found in CWI signaling pathway, and is much more important than the other components. Loss of *PKC1* results in more severe growth defects than deletion of the other members of the MAP kinase cascade (Lee & Levin 1992; Levin & Bartlett-Heubusch 1992; Levin et al. 1990). *Δpkc1* mutants lose viability in normal growth conditions but can be rescued by the osmotic support.

Pkc1 protein sequence presents two homologous regions, HR1A and HR1B, these in its N-terminal domain. HR1A region is responsible for binding to Rho1 (Schmitz et al. 2002). There is also a cysteine-rich domain (C1 domain) flanked by two imperfect C2 domain in Pkc1. C1 domain is responsible for the Rho1 binding as the HR1A domain, and the C2 domain is for phosphatidylserine binding. All C1, C2 and HR1A regions of Pkc1 are involved in the activation of Pkc1 by the phosphatidylserine/Rho1 (Mellor & Parker 1998).

The Pkc1 protein is localized at the bud tips in the budding cells and the bud neck in the mother-daughter cells which is similar to the localization of the Rho1 (Andrews & Stark 2000). The HR1 domains are responsible for the localization of the Pkc1 in the bud tip and the

bud neck. The localization of Pkc1 changes to the mitotic spindle upon removal of the HR1 domains (Denis & Cyert 2005).

In addition to the Bck1 MAPKK kinase, which is the substrate of Pkc1 in the CWI pathway, other substrates of Pkc1 have been identified. There are some targets of Pkc1 in the cell wall. Oligosaccharyl transferase is one of Pkc1 substrate. It can catalyzes protein N-glycosylation which is important for the cell wall assembly (Zufferey et al. 1995). The subunit Stt3 of Oligosaccharyl transferase can interact with the Pkc1. And the $\Delta pkc1$ shows a 50% reduced oligosaccharyl transferase activity (Yamochi et al. 1994; Park & Lennarz 2000). The chitin synthase 3 (Chs3), which is responsible for the major part of cell wall chitin deposition, is a phosphoprotein, and Pkc1 function is required for *in vivo* phosphorylation of Chs3. Apart from those cell wall related substrates, there are also some nuclear functions for Pkc1 such as arrest of secretion, mitotic recombination, G₂/M progression and the SPB duplication (Levin 2005). And Pkc1 is also the target of staurosporine action in yeast

Pkc1 can activate a basic three-protein kinase module involving an integration of the MEK-kinase, the redundant MEK-kinases Mkk1p and Mkk2p, and the MAP kinase Slt2p (Sia 1996; Irie et al. 1993; Lee et al. 1993; Lee & Levin 1992).

1.1.3.1.4 MPK1/Slt2

Several targets have been found for the Slt2 MAP kinase, including Rlm1, SBF, Ca²⁺ iron channel and the Mih1 tyrosin phosphatase (Levin 2005). Both Rlm1 and SBF are transcription factors that act in the nucleus. Rlm1 is responsible for most of the transcriptional output of CWI signaling. It can rescue the lethality of overexpression of Mkk1^{S386P} (Yashar et al. 1995; Watanabe et al. 1997). Rlm1 can interact with Slt2 in the two-hybrid system, and can be phosphorylated by this kinase (Yan & Lennarz 2002; Watanabe et al. 1995). Two phosphorylated residues of Rlm1, Ser 427 and Thr 439, are responsible of the stimulation of its activity (Jung et al. 2002). And the Rlm1 can regulate at least 25 genes, which mainly relate to the cell wall synthesis (Jung & Levin 1999). Rlm1 act as a transcriptional activator or a repressor in response to different contexts (Roberts et al. 2000). The $\Delta rlm1$ mutant displays caffeine sensitivity like the phenotype of the mutants deleted for components of the MAP kinase cascade, but deletion of *RLM1* does not cause the temperature-dependent cell lysis which is a characteristic phenotype of the MAP kinase cascade mutants. So Rlm1 is not the only target of the Slt2 (Dodou & Treisman 1997; Watanabe et al. 1995).

SBF, which contains two subunits Swi4 and Swi6, is another transcriptional factor activated by Slt2. It can bind the upstream regulatory sequence SCB (For Swi4/6 dependent cell cycle box, CACGAAA) and then activates the transcription of genes involved in G1 to S cell cycle transition (including G1 cyclins) as well as cell morphogenesis and cell wall biosynthesis. (Breedeen 2003; Moffat & Andrews 2004). To activate the transcription of the genes both the binding of SBF to the SCBS and the phosphorylation of Whi5 by Cln3-Cdc28 are required. Swi6 is phosphorylated on Ser160 during G1, and excluded from the nucleus. The phosphorylation of Swi6 is catalyzed and phosphorylated by Slt2. Swi6 is phosphorylated *in vitro* and *in vivo* by Slt2/Mpk1 in response to cell wall stress. The cell cycle related phosphorylation site of Swi6 (Ser160) resides within a consensus MAP kinase phosphorylation site. And Slt2 cannot phosphorylate the Swi6 phosphorylation site mutant (Madden et al. 1997; Baetz et al. 2001). Swi4 is the DNA binding component of SBF. It associates with Slt2/Mpk1 *in vitro* for the regulation of some cell wall related genes and morphogenesis-related genes, like *FKS2* and *PCL1* (Levin 2005). Transcriptional induction of *FKS2* and *PCL1* in response to the mild heat shock depends on Swi4 and Slt2 but not Swi6 (Baetz et al. 2001). The expression of the *FKS2* just responds to the cell wall stress, not to a specific cell cycle step. So Swi4 also plays a role in morphogenesis related the role of G1 cyclin in maintaining cell polarity (Moffat & Andrews 2004). *PCL1* and *PCL2* genes encode another G1 cyclins localized at the sites of polarized cell growth. Their overexpression can suppress the defects of $\Delta slt2$ mutant.

Bck2 that is for Bypass of C-kinase can suppress the temperature-sensitive growth defect of a $\Delta slt2$ and also a $\Delta pkc1$ mutants. The mechanism of the suppression of $\Delta slt2$ by Bck2 is shared with Swi4.

Except the two transcriptional factors Rlm1 and SBF, additional Slt2 targets exist, such as Cch/Mid1 Ca²⁺ channel, MAP kinase phosphatases, and Mih1 tyrosine phosphatase of the morphogenesis checkpoint (Levin 2005).

1.1.3.2 Activation of CWI pathway

Cell cycle regulation, heat stress, hypo-osmotic shock, pheromone-induced morphogenesis, cell wall-stressing agents, actin cytoskeleton depolarization and oxidative stress are all elements that activate the CWI Pathway (Levin 2005).

During the cell cycle regulation, Slt2 is proposed to promote bud emergence downstream of or in parallel to Cdc28, which is the CDK (cell cycle dependant kinase) of the cell cycle engine (Levin 2005). The CWI signalling is regulated through the cell cycle, peaking at time of highly polarized growth such as bud emergence. When the cell growth is polarized to a single site on the cell surface, the cell is most susceptible to lysis and cell wall stress is maximum (Levin & Bartlett-Heubusch 1992; Levin et al. 1990; Zarzov et al. 1996). Electron micrographic studies have revealed that *pkc1Δ*-arrested cells had lysed at their bud tips, which are the sites of wall remodeling (Levin et al. 1994).

CWI signaling can be activated in response to the elevated temperatures from 25°C to 39°C. Slt2 is not activated immediately upon the heat shock; its activation is detected after 20min and peaks after 30 min. By contrast, the hypo-osmotic shock can activate the CWI signaling pathway rapidly and transiently. The Slt2 is activated in 15 seconds of osmotic downshift and restored after 30 min (Kamada et al. 1995; Sohaskey 1995).

The formation of mating projections by two opposite haploids requires the polarization of the actin cytoskeleton. The CWI pathway is activated by the treatment with the mating pheromone. Pheromone-induced CWI signalling is directly linked to morphogenesis (Buehrer & Errede 1997; Errede et al. 1995). And CWI signaling defective mutants experience cell lysis during pheromone-induced morphogenesis.

There are cell wall-stressing agents, which also cause cell wall stress, like the CFW (calcofluor white), Congo red and caffeine. The CFW and Congo red have similar action mechanisms leading to the loss of the cell wall integrity. Both two can induce the phosphorylation of Slt2/Mpk1p mediated by the Mid2, and cell wall gets remodeled in response to these two agents (Nobel et al. 2000). The Congo red is a dye known to interfere with the glucan network, causing the loss of the cell wall rigidity. There are two models for the mechanism of the CR. The first one proposes that CR binds to the helical chains of β -1, 3-glucan and causes the loss of interaction between the helices. The second model proposes that CR could interfere with the cell wall components assembly enzymes (Klis et al. 2006). The calcofluor white CFW interacts specifically with chitin microfibrils in yeast and with cellulose in other organisms. Mutants isolated by their resistance to CFW are accompanied by a decreased chitin contents (Roncero & Duran 1985).

The actin cytoskeleton gets depolarized upon rapamycin treatment, which can inhibit the function of Tor1/2 protein kinase and induce Slr2 activation. And the actin antagonist latrunculin B also activates Slr2 (Harrison et al. 2001; Torres et al. 2002)

Plasma membrane stretch leads to the activation of CWI signaling pathway by physical stress. This mechanism is proposed to be the common stress generated by all activating conditions (Kamada et al. 1995)

Localization of the components of the CWI signalling at sites of polarized growth is dependent of the actin cytoskeleton and important for the pathway functioning. Under stress conditions, Rho1, Fks1, Wsc1 and Pck1 become redistributed around the cell periphery or to specific region underlying the whole cell surface. The transient delocalization of the actin cytoskeleton is thought to cause the GS and the other Rho1 effectors redistribution (Levin 2005; Andrews & Stark 2000; Delley & Hall 1999).

1.1.3.3 method for measuring the CWI pathway

The CWI pathway can be activated by a variety of external stimuli that cause cell wall stress. There are three methods for monitoring the CWI signalling. The simplest one for measuring sustained signalling through the CWI pathway is to use *lacZ* reporter gene driven by Rlm1-responsive promoters (Jung et al. 2002). The second one is to directly measure the activation state of Slr2 MAP kinase. Immuno-precipitated complexes can be used to measure the protein kinase activity of epitope-tagged Slr2 *in vitro* using bovine myelin basic protein as a substrate (Kamada et al. 1995; Zarzov et al. 1996). The third method is to use the commercially available antibodies raised against Thr²⁰²/Thr²⁰⁴ of mammalian phospho-p44/p42 Erk1 which also recognize the dually phosphorylated activated form of Slr2 (de Nobel et al. 2000; Martin et al. 2000).

1.2 the Calcineurin signalling pathway

Calcineurin signalling plays diverse roles in fungi in regulating stress responses, morphogenesis and pathogenesis. Although calcineurin signalling is conserved among fungi, recent studies indicate important divergences in calcineurin-dependent cellular functions among different human fungal pathogens. Fungal pathogens utilize the calcineurin pathway to effectively survive the host environment and cause life-threatening infections, which makes targeting calcineurin a promising antifungal drug development strategy (Thewes 2014)..

Indeed, the immuno suppressive calcineurin inhibitors (FK506 and cyclosporine A) are active against fungi.

Calcineurin (also known as protein phosphatase 2B or PP2B) is a member of calcineurin signalling pathway, a protein phosphatase under the control of Ca^{2+} and Calmodulin (CaM), plays a key role in eukaryotes. Once activated by Calmodulin, Calcineurin ensures stress-induced expression of the genes for ion transporters and cell wall synthesis through dephosphorylation of the Zn-finger transcription factor Crz1 which allows its entry into the nucleus (Stathopoulos & Cyert 1997; Matheos et al. 1997). Calcineurin also becomes essential in yeast cells lacking a functional CWI/PKC1 pathway, and vice versa: any drug or mutation affecting the Calcineurin pathway renders CWI/PKC1 pathway essential for cell survival. Thus, these two pathways are somehow connected and clearly share a common essential function. This unknown function is the subject of extensive research. At the moment, some side connections between the pathways (see below) have been found (both control the expression of FKS2, the gene encoding the alternative subunit of β -1, 3-glucan synthase and Slf2 also seems to activate the Cch1/Mid1 Ca^{2+} channel), but none of them can account for their synthetic lethality. Protein-protein interactions between Knr4 and two different subunits of calcineurin show that no significant interaction was observed with Cnb1, while a strong physical interaction between Knr4 and Cna1 was measured in the yeast two hybrid system.

1.2.1 The Calcineurin/PP2B

The Ca^{2+} calmodulin-dependent phosphatase calcineurin has been investigated in detail mainly in mammalian cells and in the yeast *Saccharomyces cerevisiae*, and has also been investigated in some lower eukaryotes like filamentous fungi (Cyert 2003; Rusnak & Mertz 2000; Thewes 2014). It regulates cellular processes as diverse as gene expression, ion homeostasis, muscle differentiation, embryogenesis, secretion, apoptosis and neurological functions. In *S. cerevisiae*, Calcineurin is not essential for growth under standard laboratory conditions, but it becomes essential for cells to adapt to a variety of environmental stresses such as high salt, alkaline pH, high temperature or the presence of mating pheromone. In mammals, alteration of Calcineurin activity has been implicated in the pathogenesis of diseases such as cardiac hypertrophy, congenital heart disease, and immunological and neurological disorders (rev. by Klee and Yang, 2007).

The importance of protein phosphorylation and dephosphorylation for numerous cellular functions has been well recognized. Protein phosphorylation by protein kinases is crucial for signal transduction, and protein phosphatases and reversible phosphorylation are equally important and critical for the integration of cellular events in fungi. Among the two major families of serine/threonine phosphatases, the regulatory role of calcineurin, a Ca^{2+} /calmodulin (CaM) regulated serine/threonine protein phosphatase is emerging. Calcineurins is a metalloenzyme with a binuclear metal center made of Fe^{3+} and Zn^{2+} (Rusnak & Mertz 2000; Hemenway & Heitman 1999). Calcineurin is a heterodimer consisting of two subunits, the α -catalytic subunit, termed calcineurin A which is about 60 kDa and binds to CaM, and a regulatory β -subunit, termed calcineurin B which is about 19 kDa (Rusnak & Mertz 2000). Calcineurin homologs are widely distributed in different organisms, ranging from parasites, fungi, plants and invertebrates to the higher vertebrates (Thewes 2014; Juvvadi et al. 2014). In mammalian cells, CnA α , CnA β , and CnA γ as three calcineurin catalytic subunits with two calcineurin regulatory subunits CnB1 and CnB2 have been identified (Rusnak & Mertz 2000). They have been implicated in diverse processes, like neuronal metabolism, T-lymphocyte proliferation, immune suppression through its downstream transcription factor NFAT (nuclear factor of activated T cells), and also in the regulation of intracellular Ca^{2+} release channels(Williams & Gooch 2012; Heineke & Ritter 2012; Musson et al. 2012). Calcineurin is the only phosphatase recognized in fungi that is regulated by Ca^{2+} and CaM and its principal target Crz1 which is homolog of NFAT has also been well studied in *Saccharomyces cerevisiae* (see below).

1.2.2 The Calcineurin-Crz1 Signalling

Crz1 was first identified as a calcineurin target in the yeast *S. cerevisiae* and its orthologues have been identified in various lower eukaryotes (Thewes 2014; Stathopoulos & Cyert 1997). Crz1 and all of its orthologues harbor C2H2 zinc finger DNA binding motifs whose number can range from one to four. In different organisms, the calcineurin-Crz1 signaling cascade can be activated by different external stimuli factors (Ca^{2+} , Mg^{2+} , Mn^{2+} , Li^+ , K^+ , and Na^+ , ethanol, caffeine, temperature, pH, blue light, the nature of the nitrogen source, and anti-fungal drugs which can increase the intracellular Ca^{2+} concentration.

In *S. cerevisiae*, the Ca^{2+} ions can enter the cytoplasm by several ways: from the external environment through the plasma membrane Ca^{2+} channel complex Mid1/Cch1 or from

intracellular calcium stores (such as the endoplasmic reticulum, the vacuole via the cation channel Yvc1 or the mitochondria). Increasing intracellular Ca^{2+} concentration leads to the activation of calmodulin, which activates calcineurin. And then calcineurin dephosphorylates its target proteins such as Crz1 (Matheos et al. 1997; Thewes 2014; Stathopoulos & Cyert 1997). Through the importin Nmd5 the dephosphorylated transcription factor Crz1 can enter the nucleus finally and can bind to its target promoters (Polizotto & Cyert 2001). The exportin Msn5 is required for the export of transcription factor Crz1 from the nucleus after phosphorylation (Boustany & Cyert 2002). It has been shown that in *S. cerevisiae* Crz1 alternates between the cytosol and the nucleus. The residence time of *S. cerevisiae* Crz1 in the nucleus is usually about 2 min after external calcium addition. And it has been shown that the calcium concentration controls the frequency of nuclear localization of Crz1, but not the duration (Cai et al. 2008). Such frequency-modulated gene expression has been discovered as a striking new mechanism and cells can coordinate their response to a signal by this new mechanism. Whereas induction of the calcineurin pathway by blue light leads to permanent nuclear localization of Crz1 after 10 min, like the nuclear localization of NFAT in mammals, which moves into the nucleus 3 to 9 min after stimulation (Bodvard et al. 2013; Kwon et al. 2008). Nevertheless, the initial response of the yeast cells after Crz1 activation is proportional to the strength of the external signal (Ca^{2+} concentration and light intensity, respectively (Bodvard et al. 2013; Cai et al. 2008). Some findings seem to indicate that different signals lead to different calcium signatures in *S. cerevisiae* as they do in filamentous fungi and it is possible that it lead to different nuclear localization patterns of Crz1 (Figure 3) (Thewes 2014).

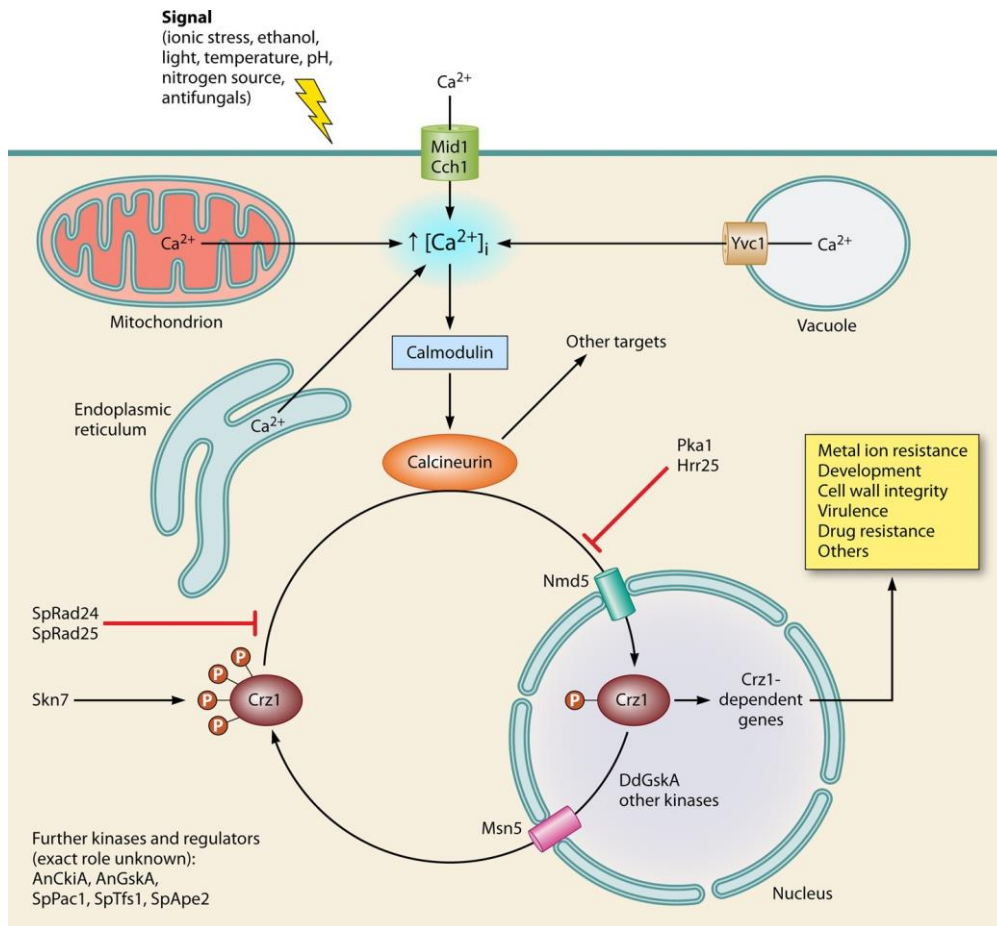


Figure 3: The calcineurin-Crz1 signaling pathway.

When the cytosolic Ca^{2+} concentration increases, calmodulin activates calcineurin, which in turn dephosphorylates Crz1. Crz1 is then imported into the nucleus and induces or represses expression of its target genes. After phosphorylation (P), Crz1 is exported from the nucleus. Several regulators of this pathway have been identified. (Thewes 2014)

1.2.3 The regulator of Crz1

A regulator of Crz1 identified in *S. cerevisiae* is the eukaryotic response regulator Skn7. It has been proposed that Skn7 protects Crz1 from degradation by binding to it and to calcineurin (Williams & Cyert 2001). Several kinases that rephosphorylate mammalian NFAT have been identified. Among them are casein kinase I glycogen synthase kinase 3 (GSK3), dual-specificity tyrosine phosphorylation-regulated kinase (DYRK), and the mitogen-activated protein (MAP) kinases p38 and Jun N-terminal protein kinase (JNK) (Thewes 2014). In contrast, the kinases that phosphorylate Crz1 preceding its nuclear export have not been identified. It is known that the casein kinase I homologues Hrr25 and Pka1 phosphorylate

Crz1 in *S. cerevisiae*, but it is assumed that phosphorylation by Hrr25 and Pka1 inhibits the entry of the protein into the nucleus (Kafadar & Cyert 2004; Kafadar et al. 2003). The exact mechanism by which Crz1 and its orthologues are exported from the nucleus is not fully understood.

1.2.3.1 The DNA binding regions of the Crz1

Crz1 can bind to 24-bp regions in target gene promoters named CDREs (calcineurin-dependent response elements) in the nucleus. And the core consensus site for Crz1 binding was shown to be GNGGC(G/T)CA (Stathopoulos & Cyert 1997; Yoshimoto et al. 2002).

1.2.3.2 Genes regulated by Crz1

Several studies used genome-wide expression analyses to investigate which genes are regulated by Crz1 and its orthologues (Thewes 2014). 116 *S. cerevisiae* genes have been identified as regulated by Crz1 (Yoshimoto et al. 2002). The majority of the Crz1-dependent genes contain one to six copies of CDRE motifs in their promoter regions. It means that these genes are likely to be direct transcriptional targets of Crz1. In *Candida albicans* there are 65 Crz1 dependent genes and 87 genes have been identified in *Candida glabrata*. In *C. albicans*, potential CDREs were also detected in the promoter regions of Crz1-dependent genes (Thewes 2014; Chen et al. 2012; Karababa et al. 2006).

Crz1 regulates between 30 and 200 genes in the different species. Common functional categories of Crz1-dependent genes range from signalling (including different transcription factors) through ion homeostasis, degradative enzymes, and cell wall biosynthesis to various genes involved in nutrient utilization of substrates (Thewes 2014). Crz1 can thereby act as an inducer or repressor of gene expression. Almost all Crz1-dependent genes are also calcineurin dependent. But It has been shown in *C. glabrata* that 38% of Crz1-dependent genes were calcineurin independent, indicating that proteins other than calcineurin might regulate Crz1 (Chen et al. 2012; Thewes 2014).

1.2.4 Cross talk with other signalling pathways

The calcineurin-Crz1 signalling pathways can cross talks with other signalling pathways. The best-investigated pathways showing a cross talk with calcineurin are the high osmolarity glycerol (HOG) pathway, alkaline stress response pathway, and the cell wall integrity (CWI)

pathway (see above).

For the alkaline stress response pathway in *S. cerevisiae* and *C. albicans*, The alkaline pH activates the Rim101 signal transduction pathway and alkaline pH leads to an increase in the cytosolic Ca^{2+} concentration, activating the calcineurin-Crz1 signalling pathway (Lev et al. 2012; Chen et al. 2011) .

The HOG pathway is one of five mitogen-activated protein kinase (MAPK) pathways in *S. cerevisiae* (Gustin et al. 1998). There are studies showing the possible interaction between the HOG pathway and calcineurin-Crz1 in *S. cerevisiae* (Shitamukai et al. 2004; Maeta et al. 2005). Direct activation of the calcineurin-Crz1 pathway in parallel to the HOG pathway has been observed, and it has been shown that calcineurin-Crz1 antagonizes the HOG pathway by downregulating it. However, the exact mechanism of the downregulation of the HOG pathway by calcineurin-Crz1 is not known.

The CWI pathway is the second of the five MAPK pathways in *S. cerevisiae*. This pathway is highly complex and interacts with the other stress response pathways (Levin 2011). One of these cross talk is with the calcineurin-Crz1 pathway which induces the *FKS2* gene encoding an alternative stress-induced beta-1,3-glucan synthase subunit. And calcineurin-Crz1 induces other cell wall related genes in different organisms. So Crz1 plays a important role, together with the CWI pathway, in maintaining cell wall integrity.

1.3 Morphogenesis and polarity in the yeast cell cycle life

It has long been recognized that yeast cell shape is correlated with cell-cycle progression. It follows that morphogenesis and the cell cycle are somehow coordinated, and numerous subsequent studies have established that the core cell-cycle machinery both regulates morphogenetic events and is in turn regulated by progression of cell morphogenesis (Review Howell & Lew 2012). And during the cell cycle, the polarity is a fundamental property of cells which alternate between states of range from tightly polarized apical focused growth to non-focused isotropic growth (Pruyne & Bretscher 2000).

1.3.1 Morphogenesis is controled by the cell-cycle

The life cycle of *S. cerevisiae* can alternate between diplophase and haplophase, which both can exist as stable cultures. Haploid cells have two mating types (**a** and α) in heterothallic

strains. Mating of **a** and α cells results in **a/α** diploids that can undergo meiosis. The four haploid products resulting from meiosis of a diploid cell are contained within the wall of the mother cell (the ascus). Digestion of the ascus and separation of the spores by micromanipulation yield the four haploid meiotic products. (YEAST molecular biology)

There are four major morphogenetic events of the cell cycle that have been identified by the early studies: The first one is polarization of the cytoskeleton and secretion in late G1 which leads to bud emergence. The second, the apical-isotropic switch in early G2, which is a depolarization of growth within the bud leading to uniform bud expansion. The third, a breakdown of mother-bud asymmetry in growth is occurring in late mitosis. All the growth is directed to the bud before this breakdown, and after all growth is evenly directed to both mother and bud. The last is refocusing of growth toward the neck upon mitotic exit that leads to cytokinesis and cell separation (Figure 4).

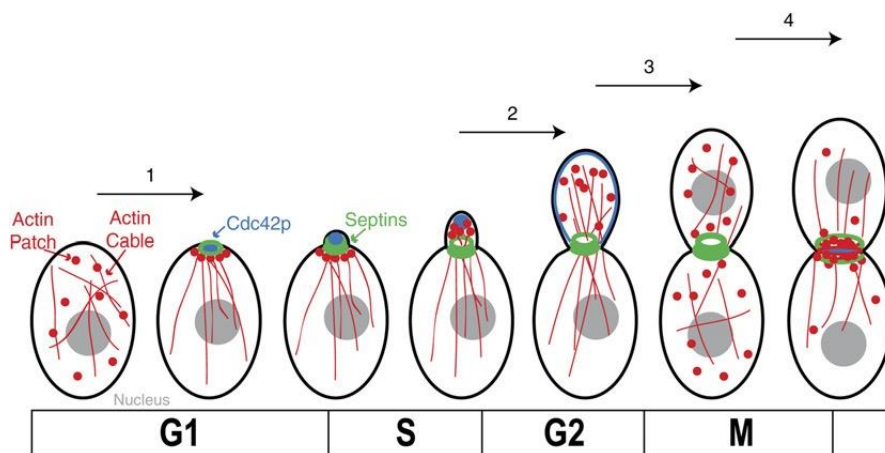


Figure 4: Morphogenetic events of the cell cycle.

Morphogenetic events of the yeast cell cycle. The four major morphogenetic events are (1) polarization in late G1, triggered by Cln1,2p-Cdc28p; (2) the apical-isotropic switch in early G2, triggered by Clb1,2p-Cdc28p; (3) breakdown of mother-bud asymmetry in late mitosis (trigger unknown); and (4) refocusing of growth toward the neck following mitotic exit, triggered by Clb-Cdc28p inactivation. Actin (red), septin (green), and Cdc42p (blue) localization during the cell cycle is indicated. (Howell & Lew 2012)

1.3.2 Polarization in yeast during the cell cycle

S. cerevisiae polarizes growth to direct budding during cell replication, and to direct shmoo formation during mating (Figure 5). The secretory pathway is important for growth to be polarized, to deliver these enzymes and membranes to the growth sites at the cell surface that is discrete. It has been confirmed that the actin cytoskeleton alone targets the secretory

vesicles that support growth. The yeast actin cytoskeleton can polarize intracellular structures during cell growth (for a review Pruyne & Bretscher, 2000). In yeast, filamentous actin is organized primarily into cortical patches and actin cables. Cortical patches are discrete F-actin- rich bodies, whereas actin cables are long F-actin bundles (Amberg 1998). Both structures lie at the cell cortex and are polarized in a cell-cycle-dependent manner.

In *Saccharomyces cerevisiae*, the actin cytoskeleton is the strength that directs polarized cell growth. And it enables the vesicular transport of cellular components towards the growing end of the cell along its cables during phases of polarized growth. Thus, actin cytoskeleton organization is crucial for morphogenesis. This complex process involves many proteins. Interestingly, several of these proteins, including Actin itself, have been isolated as “partners” interacting with Knr4 either physically or genetically.

The cell cycle begins in G1 with establishment of a nascent bud site at which the cortical patches and actin cables converge (Figure 5). Cortical patches initially cluster at its tip, cables extend from the mother cell into the bud and the bud grows apically from the tip during bud emergence.

In vegetatively growing yeast, patches and cables within the bud redistribute randomly while cables in the mother cell still extend to the bud neck. A pseudo-filamentous morphology can be induced in some *S. cerevisiae* strains by a variety of conditions. And this filamentous morphology prolongs apical growth to generate highly elongated cells (Figure 5). Cortical vegetative bud growth, the cortical patches and actin cables redistribute randomly in the mother and bud while a cytokinetic F-actin ring assembles at the bud neck, contracts and disassembles (Field et al. 1999). During the cytokinesis, Patches and cables repolarize to the former bud neck for directing the synthesis of cell walls between the two new cells. All growth is directed towards the bud during the bud formation. When growth is depolarized, bud enlargement ceases altogether and mother cells grow into huge round unbudded cells (Pruyne & Bretscher 2000).

During mating, actin also polarizes growth in the yeast. Haploid yeast cells secrete pheromones to elicit a mating response in cells of the opposite mating type. Stimulated cells become arrested in G1, express proteins necessary for cell fusion and orient growth toward mating partners by polarizing their actin cytoskeleton depending the pheromone concentration gradient (Kron & Gow 1995). The mating projections (shmoos) from two mating partners

eventually fuse and form a diploid zygote.

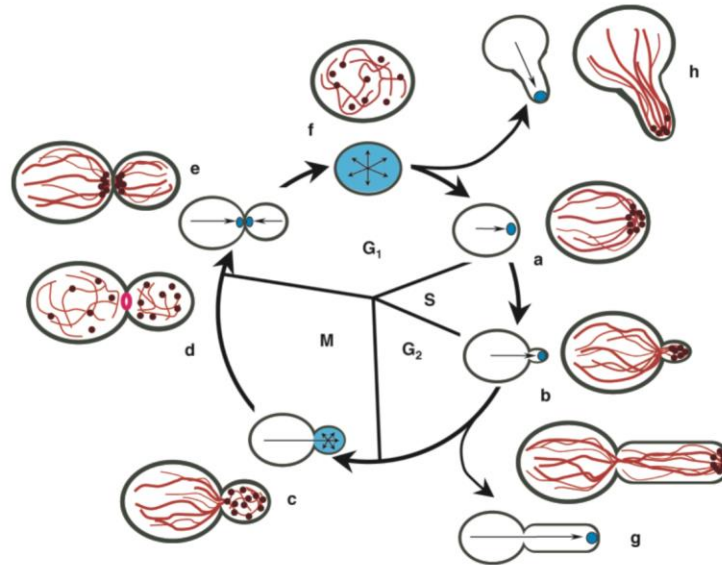


Figure 5: Cell polarity in budding yeast.

Cell polarity in budding yeast is established by the localized plasma membrane recruitment of the Rho GTPase Cdc42p (blue) and proteins related to its function. These proteins orient the actin cytoskeleton, which consists of actin cables (pink) and cortical patches (brown). In turn, the actin cytoskeleton guides secretory vesicles to the cell surface, where they accumulate (also blue) and fuse, thus polarizing growth (arrows). (Howell & Lew 2012)

1.3.2.1 Polarity establishment in G₁

Bud emergence is dependent on G₁ CDK activity and the CDK activation is the regulatory trigger for this event. The major drivers for bud emergence are the Cdc28p cyclins Cln1p and Cln2p, with some assistance from the Pho85p cyclins Pcl1p and Pcl2p (Measday et al. 1994; Moffat and Andrews 2004).

Most of the key regulators of cell polarity in yeast led to a hierarchical model for polarity establishment. And the master regulator Cdc42p orients the cytoskeleton for bud growth. At the top of the hierarchy is a set of “bud-site selection” proteins.

The set of “polarity establishment” proteins is centered on the conserved Rho-family GTPase Cdc42p. Both Cdc42p and its GEF Cdc24p are absolutely required for polarized organization

of the cytoskeleton and for bud emergence. Cdc42p is concentrated in a patch at the presumptive bud site (Richman et al. 1999; Richman et al. 2002) and regulates a variety of “effector” proteins to bind specifically to GTP-Cdc42p. The localization of Cdc42p (GTP-Cdc42p) is assumed to be critical to establish polarity (Howell & Lew 2012). Localization of Cdc42p could occur through interaction with a prelocalized anchoring structure. Polarization can occur at random sites presumed to lack prelocalized anchors. Thus, it is thought that Cdc42p can become clustered at a nascent polarization site and remain clustered, despite diffusion, without needing to be anchored to a stable structure (Slaughter et al. 2009; Wedlich-Soldner et al. 2004).

It is assumed that Cdc42p concentration was triggered by prior localization of its GEF to a site marked by cellular landmarks. But *rsr1*Δ cells still pick one and only one (randomly located) bud site with apparently normal timing and efficiency. This process which is called symmetry breaking suggests that there is a positive feedback loop or amplification mechanism that allows a stochastic fluctuation in polarity factor concentration at some random site to promote accumulation of more polarity factors at that site (for a review Howell & Lew, 2012). Polarization of Cdc42p in *rsr1*Δ cells does not require polymerized actin or microtubules (Iraoqui et al. 2003). GTP-Cdc42p at the membrane can (via PAK interaction) recruit a PAK- Bem1p-GEF complex that can then via GEF activity, which may be stimulated by Bem1p interaction (Shimada et al. 2004). When a polarization site with concentrated GTP-Cdc42p is established, actin cables are oriented toward the site, actin patches which are sites of endocytosis (Kaksonen et al. 2003).

Cytoskeletal polarity is guided by the distribution of Cdc42p and its GEF Cdc24p on the plasma membrane in yeast. The distribution of Cdc24p-Cdc42p complexes ranges from a tight polarization during bud emergence through a cap-like distribution during apical growth to a diffuse distribution during isotropic growth. The initial polarization of Cdc24p and Cdc42p during shmoo-site (see below) and bud-site selection depends strongly on Bem1p. Bem1p colocalizes with Cdc24p and Cdc42p to growth sites, and its transcription in G1 coincides with these early polarization events (for a review Pruyne & Bretscher, 2000)

Bem1p is also not essential for forming a nascent bud site, although it does greatly facilitate bud emergence and is required for normal bud morphology. Bem1p might facilitate Cdc24p-Cdc42p clustering by binding to other proteins, such as F-actin and Ste20p, that cross-link Bem1p-Cdc24p-Cdc42p complexes (Leeuw et al. 1995). It remains to be determined whether

the Cdc24p-Cdc42p clustering that occurs in *bem1Δ* cells is mediated through a self-association between Cdc24p-Cdc42p complexes that is activated at START, or whether additional proteins are involved. Cdc24p and Cdc42p continue to remain clustered for apical growth during shmoo formation, early vegetative bud growth, and filamentous bud elongation.

A group of polarity-determining proteins that comprises Bni1p, Sph1p, Spa2p, Pea2p and Bud6p (Aip3p) function as an apical scaffold for Cdc24p-Cdc42p during these processes. Spa2p, Pea2p and Bud6p have been detected in a 12S complex termed the polarisome (Sheu et al. 1998) and we will refer to them collectively here as polarisome proteins. Polarisome proteins are required for apical actin organization. In their absence, vegetative buds grow as spheres rather than ellipsoids, filamentous bud elongation is blocked and shmoo growth depolarizes to generate short and broadened projections (Amberg et al. 1997; Evangelista et al. 1997; Mösch & Fink 1997). Polarisome mutants also have widened mother-bud necks (Zahner et al. 1996)

It is suggested that the polarisome links RhoGTPase signaling to actin filament assembly by protein-protein interactions involving polarisome components. The polarisome links RhoGTPase signaling to actin filament assembly. Bni1p is central to these interactions, binding Bud6p and Spa2p, as well as activated RhoGTPases, which contains Cdc42p, Rho1p, Rho3p, and Rho4p. Spa2p, Sph1p and Pea2p localize to growth sites and provide a polarized docking site for Bud6p and Bni1p (Roemer et al. 1998; Evangelista et al. 1997; Amberg et al. 1997; Arkowitz & Lowe 1997; Fujiwara et al. 1998; Sheu et al. 1998). Bni1p binds profilin (Pfy1p) that is a protein that stimulates actin polymerization and Tef1p/Tef2p that is an actin-bundling protein. Whereas Bud6p binds to actin filaments (Amberg et al. 1997; Umikawa et al. 1998; Imamura et al. 1997).

1.3.2.2 Polarity establishment in Shmoo

External pheromone can guide polarized shmoo growth during mating. Pheromone stimulation activates G-protein-coupled receptors that generate free Gβγ, which in turn recruits a polarity determinant Far1p to the plasma membrane. Gβγ and Far1p and then together recruit Bem1p, Cdc24p and the PAK Ste20p to assemble a Cdc42p-dependent signalling complex (Butty et al. 1998; Leeuw et al. 1995; Nern & Arkowitz 1999). External pheromone gradients lead to a higher concentration of Cdc42p associated with the plasma membrane to one side of the cell. Actin-dependent clustering of pheromone receptors further

tightens these signalling complexes into a patch directed toward the pheromone source (Ayscough et al. 1997). This patch then orients the actin cytoskeleton and directs shmoo growth.

1.3.2.3 Control of Cdc28p by the Morphogenesis Checkpoint

In response to insults that impair bud formation or affect the actin cytoskeleton, *S. cerevisiae* undergoes a mitotic delay known as the Morphogenesis Checkpoint (Sia et al.,1996), which avoids formation of bi-nucleated cells. This delay is mediated by the inhibitory action of the Swe1 (budding yeast homolog of Wee1) tyrosine kinase on the Cdc28 CDK (homolog of mammalian Cdc2). Activation of the checkpoints results in cell cycle arrest in G2. The phosphorylation of Cdc28 can be reversed by the action of Mih1 phosphatase (homolog of Cdc25). Both Slt2 MAP kinase and Calcineurin are involved in this checkpoint: Slt2 promotes checkpoint activation by down regulating the Mih1 phosphatase, while Calcineurin multilaterally activates Swe1 at the transcriptional, post-translational and degradation level (Miyakawa and Mizunuma, 2007).

1.4 Knr4

The *knr4* (killer nine resistant 4) mutant was first selected because of its resistance to K9 killer toxin from *williopsis saturnus var: mrakii* IFO 0895. *knr4* mutants display additional cell wall defects, which are osmotic sensitivity; sensitivity to cercosporamide (a known antifungal agent) ; and resistance to the (1,3)- β -glucanase Zymolyase; In addition, this gene is a multicopy suppressor of several Calcofluor white hyper-sensitive mutants (Martin et al. 1999). The *KNR4* gene encodes a protein containing 505 amino acids whose calculated molecular mass is 57,044KDa. (Hong, Mann, Brown, et al. 1994).

1.4.1 The localization of the Knr4p

1.4.1.1 During vegetative growth

A fusion of Knr4p to the green fluorescent protein GFP is localized at the presumptive bud site in unbudded cells, distinct from the nucleus, and then at the bud tip in cells with small buds. During further growth of the daughter cell, the GFP-Knr4 fusion appears as a patch at the neck between mother and daughter cells. Before nuclear division, the fusion protein patch

separates into two parts, one migrating to the cytoplasm of the daughter cell and the other remaining in the mother cell (Martin et al. 1999).

1.4.1.2 Knr4 localization during shmoo formation

Knr4 protein localizes to a discrete bright spot near the cell shmoo tip (Dagkessamanskaia et al. 2010). This confirms the observations of a recently published global analysis of proteins localized at the shmoo tip, which highlighted the fact that most of proteins localized at the bud-tip during vegetative growth are found at the shmoo tip during mating (Narayanaswamy et al. 2009).

1.4.2 The function of the *KNR4*

1.4.2.1 *KNR4* involved in the cell wall (1, 3) - β - glucan and (1, 6) - β - glucan synthesis

KNR4 was originally selected by restoring wild type sensitivity to K9 killer toxin of a resistant *knr4* mutant. The *knr4* mutant also displays resistance to the (1, 3) - β - glucanase. And the disruption of the *KNR4* locus leads to reduced both (1, 3) - β - glucan synthase activity and (1, 3) - β - glucan content in the cell wall. (Hong et al, 1994). HPLC analysis have shown that both (1, 3) - β - glucan and (1, 6) - β - glucan are reduced in the mutant *knr4 Δ* . Amount of (1, 3) - β - glucan in *knr4 Δ* is reduced to almost half of the level measured in wild-type strains. And there is a four-fold reduction in the amount of (1, 6) - β - glucan synthesized in the mutant strain compared to the wild-type strains.

1.4.2.2 *KNR4* is a suppressor of *Saccharomyces cerevisiae* Calcofluor-white hypersensitive mutants and is involved in the transcriptional control of chitin synthase

The *KNR4* gene has been identified as a suppressor of *Saccharomyces cerevisiae* calcofluor-white-hypersensitive mutants (cwh), which are often characterized by increased chitin levels. The suppressor effect of *KNR4* gene works by lowering the content of this polymer to levels closer to wild-type without affecting the β -glucan and mannan. The chitin synthesis is governed by *CHS1*, *CHS2* and *CHS3* genes encoding the three membrane-bound chitin synthetases. And the suppressor *KNR4* can reduce the mRNA level of *CHS1*, *CHS2*, *CHS3* (Hong, Mann, Shaw, et al. 1994b; Martin et al. 1999).

It is also reported that there is four times more chitin in the *knr4Δ* mutant cells compared to the wild-type cells (Hong, Mann, Shaw, et al. 1994b; Martin et al. 1999).

1.4.2.3 Knr4p is a member of the PKC1 / CWI signalling pathway

The PKC1 pathway, also named CWI for “cell wall integrity” signalling pathway, is a linear cascade of Mitogen Activated Kinases (MAP Kinases) under the control of Pkc1, the budding yeast homolog of mammalian Protein Kinase C protein. This pathway is regulated periodically through the cell cycle, peaking at the time of bud emergence, the time at which growth is most highly polarized (see 1.1)

Different results have established the links between *KNR4* and the PKC1 pathway (Figure 6). First, *KNR4* genetically interacts with *BCK2* which stands for “Bypass of Kinase C- 2”. Products of these two genes participate in the expression of the three G1 cyclins at the G1-S transition (Martin-Yken et al. 2002). Second, a physical interaction between Knr4 and the MPA kinase Slr2 has been demonstrated by several complementary methods. This interaction is proposed to modulate Slr2 activation of transcriptional factors Rlm1 and SBF (Martin-Yken et al, 2003). Last, the presence of Knr4 protein in the cell, and notably with its N-terminal domain, is absolutely necessary for cell viability in the absence of a functional Pkc1-dependent cell integrity pathway (Durand et al. 2008; Adilia Dagkessamanskaia, Durand, et al. 2010)

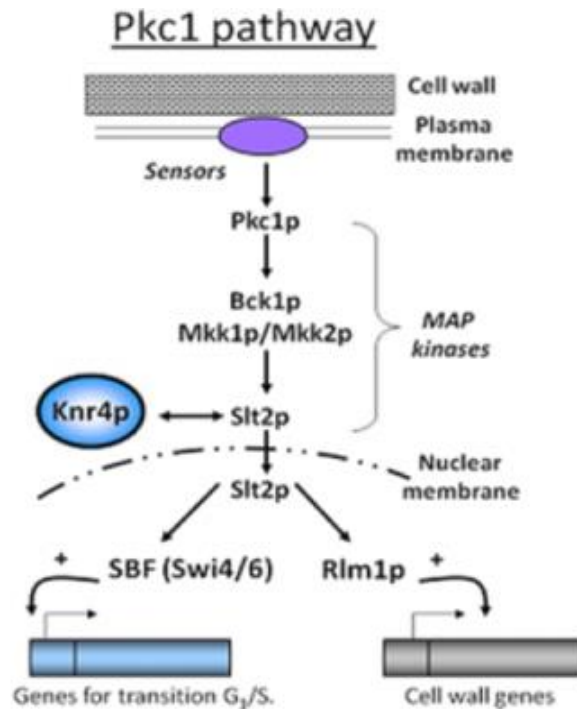


Figure 6: Schematic representation of the PKC-SLT2 pathway and possible position of Knr4 in this pathway (Durand *et al.*, 2008)

1.4.2.4 Knr4 involved in the Calcineurin pathway

Calcineurin, also called Protein Phosphatase 2B (PP2B) is a protein phosphatase under the control of Ca²⁺ and Calmodulin (CaM), which plays a key role in eukaryotic cells by regulating cellular processes as diverse as gene expression, ion homeostasis, muscle differentiation, embryogenesis, secretion, apoptosis and neurological functions (see above, 1.2 calcineurin pathway). Once activated by the upstream phosphatase Calmodulin, Calcineurin dephosphorylates the Zn-finger transcription factor Crz1 that allows its entry into the nucleus where it ensures stress-induced expression of the genes coding for ion transporters and cell wall synthesis (Matheos *et al.*, 1997, Stathopoulos and Cyert, 1997). Calcineurin also becomes essential in yeast cells lacking a functional PKC1 pathway, and vice versa: any drug or mutation affecting the Calcineurin pathway renders PKC1 pathway essential for cell survival. Thus, these two pathways are connected and clearly share a common essential function. This unknown function is the subject of extensive research. At the moment, some side connections between these two pathways have been found: both control the expression of *FKS2*, the gene encoding the alternative subunit of β (1,3)-glucan synthase, and Slt2 also seems to activate the Cch1/Mid1 Ca²⁺ channel, but none of these connections can account for their synthetic lethality. Protein-protein interactions between Knr4 and two different subunits

of Calcineurin show that no significant interaction was observed with the regulatory sub-unit Cnb1, while a strong interaction was detected between Knr4 and the catalytic subunit Cna1 (Adilia Dagkessamanskaia, El Azzouzi, et al. 2010).

1.4.3 The structure of the Knr4 and the role of the different domains of Knr4

The recent discovery that protein domains or even complete proteins lack 3D structure at the native state and nevertheless carry biological activity resulted in them being classified as **Intrinsically Disordered** (or Unstructured) **Proteins, IDP** or IUP (Dunker et al. 2001; Tompa 2002). Proportion of disordered proteins is rather independent on the proteome size, with all eukaryotes having about 32% or more of disordered proteins (Xue et al. 2010). IDPs are involved in numerous biological processes (Dunker et al. 2008)(Dunker et al. 2002) and their functions often rely on interactions with specific binding partners contributing to their regulation or to the assembly of supra-molecular complexes. Biochemical, biophysical tools and *in silico* analysis have established that Knr4 protein is an Intrinsically Disordered Protein “IDP” or Intrinsically Unstructured Protein “IUP”. Knr4 is composed of a functional central globular core, a poorly structured N-terminal and a large natively unstructured C-terminal domains (Durand et al. 2008) .

In silico analysis based on amino acids composition profiling of the Knr4 is similar to typical intrinsically disorderd proteins (IDPS) whose features are presence of numerous charged groups, large net charge at neutral pH, depletion in the order-promoting amino acids residues (I, L, V, W, F, Y, C and N), and enrichment in the disorder-promoting amino amino acids (as E, K, R, G, Q, S and P). It shows low secondary structure content and a high probablility of coiled coils whithin the highly disordered C-terminus (340-505). The N-terminus (1-80) is predicted to be disordered also, whereas the central core (80-340) is predicted to be compact, folded and globular(Durand et al. 2008) .

Secondary structure of the full length Knr4 and various fragments was also examined by far - UV circular dichroism (CD). It validated the disordered state of the full length Knr4 protein. Remarkably, this analysis also showed that the C-terminal domain becomes partially folded in the presence of other Knr4p fragments. Similarly the N-terminal (1-80) part also appeared folded in the context of Knr4 (1-340) upon this particular analysis (Durand et al. 2008).

SDS-gel electrophoresis analysis indicates that the C-terminal tail is largely responsible for the aberrant migration of Knr4 on SDS-PAGE. The central core of Knr4 migrates at the expected size on SDS-PAGE and thus is likely folded. Moreover, it appears less accessible during the immunization as shown by the fact that it is not recognized by polyclonal Antibodies raised against the whole protein. We thus believe this central core to be “covered” by the highly flexible N- and C-terminal domains (Durand et al. 2008).

Combining the far-UV CD data and the SDS-PAGE analysis of trypsin treatment to Knr4 and its variant fragments, it indicated that this polypeptide possesses hydrodynamic properties typical of a natively unfolded protein. And the formation of the coiled-coil oligomers depends on the presence of the remaining parts of the protein (Durand et al. 2008).

1.4.3.1 The N-terminal domain of Knr4 and its cellular role

1.4.3.1.1 Role of the N-terminal domain in the protein cellular localization

The Knr4 full-length protein localizes at the presumptive bud site in G1, then at the tip of small buds and at the mother-daughter neck during cytokinesis. And the N-terminal domain has been proved to be required for the correct localization at the bud tip by the fluorescence microscopy observations. In contrast, removal of the C-terminal domain did not impair the correct localization of the Knr4 fragment (1-340) (Adilia Dagkessamanskaia, El Azzouzi, et al. 2010) .

Upon pheromone treatment of haploid cells, Knr4 full-length is localizes as a spot at the shmoo tip. And this specific localization also requires the presence of the protein N-terminal domain. This localization is lost for the protein Knr4 (80-508) that is just without N-terminus. Again, the absence of the C-terminal domain does not perturb the correct localization of Knr4 (1-340) at the shmoo tip (Dagkessamanskaia, et al. 2010) .

In the paper Dagkessamanskaia, 2010, the authors postulate that Knr4 can interact with at least one protein through its N-terminus to ensure its localization at the sites of polarized growth (bud tip and shmoo tip). Some of the identified physical interaction partners of Knr4 are proposed to play this role, such as Rvs167 (actin -binding protein) and Bud3 (involved in bud-site selection).

1.4.3.1.2 The N-terminal domain of Knr4 influences shmoo formation

It has been reported that the copy number of *KNR4* in the cell had an influence on the efficiency of shmoo formation (Martin et al., 1999). And absence of the Knr4 N-terminal domain can also slow down the shmoo formation process and causes cells to go on budding significantly longer than wild-type after pheromone exposure. Thus, it appears that Knr4 correct localization at the incipient shmoo site is necessary for the further onset of polarized growth in response to pheromone. Moreover, the delay in cell cycle arrest observed in *knr4* mutant may also be directly linked with the hyper activated and unregulated SBF transcriptional activity observed in this mutant (Martin-Yken et al., 2003) (Adilia Dagkessamanskaia, El Azzouzi, et al. 2010) .

1.4.3.1.3 N-terminal domain of Knr4 is required but not sufficient for interaction with Calcineurin

Calcineurin or PP2B is a multiproteic complex, composed of a regulatory subunit Cnb1 and a catalytic subunit, either Cna1 or Cna2 (Cyert et al. 1991; Cyert & Thorner 1992). Knr4 interacts strongly with Cna1 but no significant interaction with Cnb1 has been measured by the yeast Two-Hybrid system, and the N-terminal of the Knr4 is necessary for the interaction, although it is not sufficient to ensure the full interaction by itself. Remarkably, the interaction strength of Knr4 (1-340) with Cna1 is higher than the Knr4 full-length, which suggests that the large disordered C-term might inhibit the Knr4-Cna1 interaction (Dagkessamanskaia, et al. 2010) .

1.4.3.1.4 The Knr4 N-terminal regions is required for the protein-protein interaction

Knr4 is reported to interact with different protein partners strongly, one of which is Tys1p, a known *in vivo* protein partner of Knr4 isolated by global two-hybrid screen. And the N-terminus of Knr4 is needed for its ability to interact with this partner. Even the Knr4 (1-80) N-terminal domain alone can interact with this partner strongly. In addition to the two-hybrid interaction, *in vitro* surface Plasmon resonance test also confirmed that the N-terminal Knr4 domain is necessary for the interaction (Dagkessamanskaia, et al. 2010) .

1.4.3.1.5 The Knr4 N-terminal region is essential in the absence of a functional PKC1-SLT2 pathway

The N-terminus of Knr4 is essential in the absence of a functional *PKC1-SLT2* pathway. Knr4 (80-505), lacking the 80 N-terminal residues is totally unable to suppress the synthetic lethality of *bck1Δknr4Δ* and *slt2Δknr4Δ* double mutants. In contrast, the Knr4 (1-340) is as functional as the full-length protein. But neither the N-terminal domain nor the central core alone can rescue the synthetic lethality between *knr4Δ* and mutants of PCK1 pathway. So the N-terminal part of Knr4p needs to be linked with the central core Knr4 (80-340) fragment to be fully effective regarding bypass of PKC1 pathway (Durand et al. 2008) .

1.4.3.2 The central core of Knr4 and its role

1.4.3.2.1 The Knr4 (80-340) fragment is sufficient to complement most of the *knr4* mutant phenotypes

Our results point to Knr4 biological activity to be ensured by its central core, located within residues 80-340. Durand et al. tested the complementation ability of different fragments of Knr4 to the temperature sensitivity and cell wall-defective phenotypes of the *knr4Δ* mutant. They found that the 80 N-terminal and 165 C-terminal amino acids regions of Knr4 are dispensable for the protein ability to complement the deletion mutant under these stresses. However Knr4 (80-340) fragment is slightly less efficient than the full size protein, recovering only partially the sensitivity to Calcofluor white and the growth at 37 °C. And the N-terminal and the C-terminal domain alone are unable to complement the mutant at all. (Durand et al. 2008)

1.4.3.2.2 Central core of the Knr4 in *PKC1-SLT2* pathway

Even though Knr4 (80-340) can not compensate the synthetic lethality of the *bck1Δknr4Δ* and *slt2Δknr4Δ* double mutants, it is able to rescue *rlm1Δknr4Δ* and *swi4Δknr4Δ*. Martin-Yken et al. found that Knr4 is required for the regulation of the kinase activity of Slt2. Durand et al. speculated that the 80-340 domain is necessary for Slt2 MAP kinase to activate Rlm1, SBF and the others transcriptional factors and thus to maintain cell viability in the absence of one of them.

1.4.3.3 The C-terminal domain of Knr4 and its cellular role

1.4.3.3.1 The negative effects of the C-terminal region for the interactions with the partners

Knr4 C-terminus has an inhibitory effect of the whole protein-protein interactions. Surface Plasmon Resonance (SPR) analyses clearly demonstrated the negative influence of the unstructured C-terminus on the full protein binding capacity. Hence, it was hypothesized that this large intrinsically disordered C-terminal part of the protein is not directly involved in the protein-protein interaction, but rather serves as a negative regulator for these interactions. This could be ensured by covering and protecting the N-terminus and the central core, or simply by steric inhibition. The C-terminus could be specifically cleaved to allow the proper protein interactions to take place at the correct time or cell localization. (Adilia Dagkessamanskaia, El Azzouzi, et al. 2010)

1.4.4 Partners of the Knr4

Protein-protein interaction networks contain nodes with numerous connections, called “hub proteins”. They are divided into “party hubs” which can interact with several proteins at the same time and constitute protein complexes and “date-hub”, able to interact with several partners at different times or locations in the cell.

The conformational flexibility of IDPs gives them the ability to be involved in one-to-many binding (where a single disordered region binds to several structurally diverse partners), and the decoupling of binding affinity and specificity enables rapid association/dissociation with their partners even without high binding strength (Uversky 2013; Dunker et al. 2005). Hence, hub proteins might actually fulfill their multiple signalling functions thanks to their intrinsically disordered nature.

As reported, Knr4 appears implicated in several diverse cellular functions, interconnecting essential cellular processes such as resistance to cell-wall stress, regulation of cell-wall biosynthesis genes, cell integrity and calcium signalling pathways, cell cycle progression and morphogenesis. Moreover, Knr4 is a characterized IDP and displays more than 300 described partners (<http://www.yeastgenome.org>). It is thus a typical yeast hub protein.

Combining results obtained by the TAP-tag method, Two-hybrid test and synthetic lethal arrays analysis, a summary of the interactome network can be proposed (Figure 7).

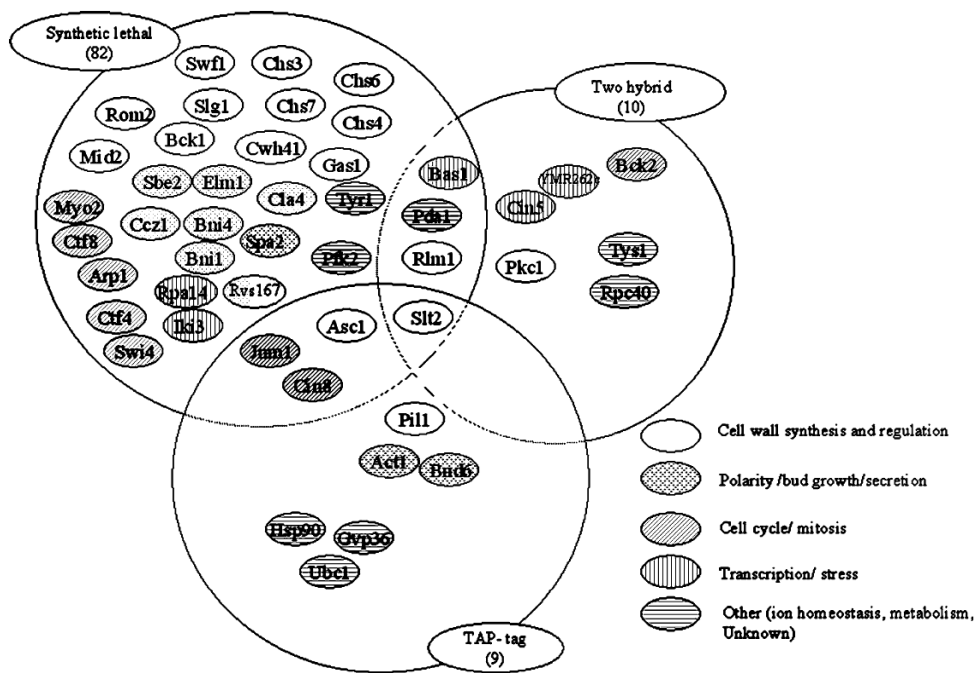


Figure 7: Overlapping of data sets from synthetic lethal, two- hybrid and TAP-tag interaction screens. (Fadi Basmaji et al. 2006)

There are 9 *Knr4* interacting proteins identified by the TAP-tag method. These 9 proteins can be divided into three categories of biological processes. The first one comprises *Slt2* and *Pil1* which are involved in the cell wall maintenance and biogenesis. *Pil1* acts to down regulate the activity of *Pkc1-Slt2* MAP kinase pathway. So the interaction of *Pil1* with *Knr4* is consistent with a regulation of *Slt2* activity. From the second category, four proteins *Bud6*, *Act1*, *Cin8* and *Jm1* have functions in cell polarity, bud emergence and spindle pole body orientation during mitosis. The last category contains *Ubc1*, *Asc1* and *Gvp36* (Fadi Basmaji et al. 2006).

180 genes show synthetic lethal interactions with *KNR4* deletion (SGD <http://www.yeastgenome.org/locus/S000003461/overview>). Some of these genes related to the cell wall synthesis, morphogenesis, transcription and metabolism are shown on (Figure 7). Among these genes are *BNII* and *BNR1* whose products are “formins”, proteins responsible for the assembly of linear actin cables respectively in the bud and at the bud neck (Pruyne D, et al. 2004). Formins are a conserved family of proteins that promote the assembly of actin filaments, a function that is necessary for remodeling of the actin cytoskeleton during such processes as budding, mating, cytokinesis, endocytosis, and in higher cells, cell adhesion and migration. Another remarkable product of a gene synthetic lethal with *KNR4* deletion is *Spa2*, a

key component of the polarisome complex, whose function is to organize actin cytoskeleton during polarized growth. Spa2 also acts as a scaffold ensuring the cellular localization of Mkk1p and Mpk1p cell wall integrity signalling components. In addition, it is a potential substrate of the cell cycle dependant kinase Cdc28.

10 proteins have been identified as Knr4 partner proteins by the Two - Hybrid method. Tys1, which shows the strongest interaction with Knr4, is an essential cellular protein, the only yeast tyrosyl tRNA synthetase required for protein synthesis. We have shown that both *KNR4* and *TYS1* are required for efficient spore formation during *S. cerevisiae* meiotic process. Moreover, the strong identified physical interaction between Knr4 and Tys1 is likely one of the molecular elements responsible for coupling chitosan and dityrosine formation during prospore wall formation. Use of the site-directed mutagenesis mutants of Knr4 suggests that phosphorylated residues S200 and S203 can influence the interaction with Tys1.

Slt2 MAP kinase is identified as a Knr4 partner by all three methods. Slt2 is the final effector of the PKC1 pathway. The Slt2p kinase undergoes phosphorylation on Thr190 and Tyr192 upon the pathway MAP Kinases cascade in various environmental conditions. The deletion of *KNR4* increases the amount and the dual phosphorylation of Slt2. In addition, the activation of Rlm1 by Slt2 kinase is reduced upon deletion of *KNR4* gene.

2.4.3 The phosphorylation residues of the Knr4 and their functions

As referred before, Knr4 is composed of a functional central globular core flanked by a poorly structured N-terminal domain and a large natively unfolded C-terminal domain. A combination of global research works from several groups revealed 18 *in vivo* phosphorylated residues in Knr4 protein, identified by different methods including mass spectrometry, electron transfer dissociation, multidimensional chromatography technology (From the <http://www.phosphogrid.org/sites/33481/YGR229C.phospho>; Albuquerque *et al.*, 2008; Chi *et al.*, 2007; Ficarro *et al.*, 2002; Li *et al.*, 2007; Smolka *et al.*, 2007, Basmaji *et al.*, 2006). These residues are located in the Knr4 protein central core and C-terminus (Figure 8).

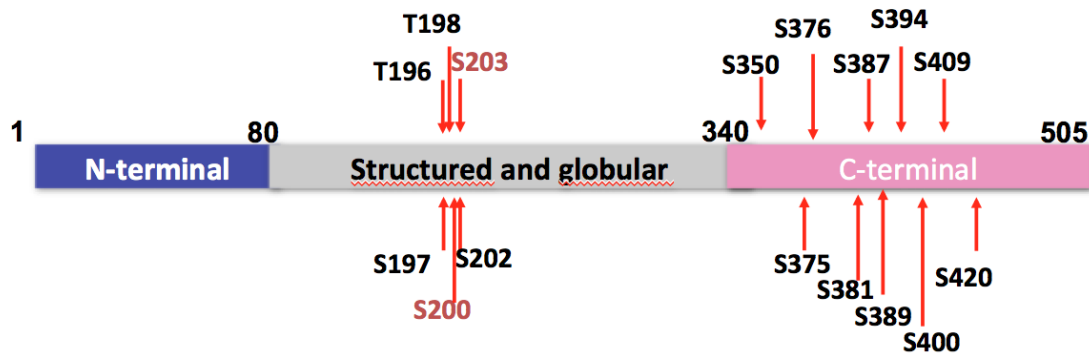


Figure 8: The phosphorylation sites of the Knr4 protein

(From the <http://www.phosphogrid.org/sites/33481/YGR229C.phospho>)

Among these 18 phosphorylation sites, there are some important sites related to the CWI pathway, calcineurin A and morphogenesis. The abundance of phosphorylation on residue S375 is 3 times down regulated in knockout of YJL095W, which is *BCK1* (CWI pathway). S389 and S394 are related *CNA1* (YLR433C), which is one isoform catalytic subunit of calcineurin (Introduction 1.2), and other two more genes *PKH3* and *PTK1*. *PKH3* (YDR466W) encodes a protein kinase with similarity to mammalian PDK1 and Pkh1p/Pkh2p, two redundant upstream activators of Pkc1p (Inagaki et al. 1999). And Ptk1 (YKL198C) that is a putative serine /threonine protein kinase involved in polyamine transport. Ptk2 that arose from the whole genome duplication is the paralog of Ptk1 (Nozaki et al. 1996; Kakinuma et al. 1995; Byrne & Wolfe 2005). S200 or S203 appear to be the targets of another member of the CWI pathway, Mkk1. And S200 might also be phosphorylated by the product of *RTK1* (YDL025C) which is a putative protein kinase potentially phosphorylated by Cdc28p. Rtk1 can interact with ribosome biogenesis factors, Cka2, Gus1 and Arc1 (Breitkreutz et al. 2010; Tkach et al. 2012; Ubersax et al. 2003; Hunter & Plowman 1997). These global phosphoproteome analysis link S200 and S203 functions related to the several genes like *YCK1*, *PSR1*, *PTC4*, *PTC6*, *YVHI*, *CKA2*, *TPK2* and *BUB1*. Yck1 (YHR135C) is palmitoylated plasma membrane-bound casein Kinase I isoform and shares redundant functions with Yck2 in morphogenesis, proper septin assembly, endocytic trafficking and glucose sensing (Robinson et al. 1992; Reddi & Culotta 2013; Robinson et al. 1993). Psr1 (YLL010C) which is a plasma membrane associated protein phosphatase, is involved in the general stress response and is required along with binding partner Whi2 for full activation of STRE-mediated gene expression, possibly through dephosphorylation of Mns2 (Sinioglou

et al. 2000; Byrne & Wolfe 2005; Kaida et al. 2002). Very interestingly, Ptc4 (YBR125C) is the Cytoplasmic type 2C protein phosphatase (PP2C), identified as a high copy number suppressor of *cnb1 mpk1* synthetic lethality. The overexpression of Ptc4 decreases high-osmolarity induced Hog1p phosphorylation and kinase activity (Cheng et al. 1999; Shitamukai et al. 2004; Huh et al. 2003). Bub1 (YGR188C), which is a protein kinase, involved in the cell cycle checkpoint into anaphase forms a complex with Mad1 and Bub3 crucial to prevent cell cycle progression into anaphase in presence of spindle damage. And CDC28-mediated phosphorylation (introduction 1.3) at Bub1p-T566 is important for degradation in anaphase and adaptation of checkpoint to prolonged mitotic arrest, through association with centromere DNA via Skp1 (Kitagawa et al. 2003; Brady & Hardwick 2000; Farr & Hoyt 1998; Nerusheva et al. 2014; Goto et al. 2011; Byrne & Wolfe 2005). Yvh1 (YIR026C) which is the protein phosphatase is involved in vegetative growth at low temperatures, sporulation and glycogen accumulation (Park et al. 1996). Cka2 is α -catalytic subunit of casein kinase 2 (CK2) (Ackermann et al. 2001; Padmanabha et al. 1990) and Tpk2 which is cAMP-dependent protein kinase catalytic subunit promotes vegetative growth in response to nutrients via the Ras-cAMP signaling pathway (Ordiz et al. 1996; Toda et al. 1987). (http://www.sbeams.org/devDC/sbeams/cgi/Glycopeptide/peptideSearch.cgi?action=Show_hits_form;search_type=accession;organism=sce;search_term=YGR229C.)

Hence, it appears clearly that several kinases and phosphatases affecting phosphorylation of Knr4 protein *in vivo* are involved in the CWI pathway, cell growth and proliferation and cell morphogenesis. Thus, we believe that the phosphorylation sites of Knr4 might be key points for its cellular function in the signalling pathway and the morphogenesis checkpoint and cell wall synthesis.

1.5 K9 killer toxin

K9 killer toxin, also called HM-1, is a small peptide produced by *Williopsis saturnus var: mrakii* IFO 0895 (previously known as *Hansenula mrakii*) and is strongly cytotoxic against *Sacharomyces cerevisiae* (Tetsuro Yamamoto et al. 1986; Teturo Yamamoto et al. 1986). The gene encoding K9, which is *HMK* gene, is from chromosomal DNA of K9 producing yeast. And *HMK* gene encodes a precursor to killer toxin of 125 amino acids, which has a N-terminal signal sequence of 37 aa which is removed upon secretion by a signal peptidase (Kimura et al. 1993).

1.5.1 Protein structure of the K9 killer toxin

K9 consists of 88 amino acid and 10 of these 88 amino acids are cysteines. It contains five disulfide bridges (S-S). K9 can be inactivated by reducing agents, like 2-mercaptoethanol and dithiothreitol. It suggested that the S-S bonds forming tertiary structures maintaining the structure and biological activity of the K9 killer toxin. The molecular mass of is 9.5kDa. (Tetsuro Yamamoto et al. 1986; Selvakumar, Miyamoto, et al. 2006).

The nmAb-KT (neutralizing monoclonal antibody) can bind the K9 and neutralise the K9 cytotoxic activity, whereas the other K9 specific monoclonal antibodies 1F1 and 4A2 can not neutralise the K9 killer toxin activity (Tetsuro Yamamoto et al. 1986; Komiyama et al. 2004). SPR analysis of K9 peptide showed the K9 peptide P6 (³⁹TGGSTDGKQG⁴⁸) has a marked binding affinity for nmAb-KT and the neighbouring peptide P5 and P7 which overlap partially with P6 have a weaker binding affinity for nmAb-KT among the 13 overlapping peptides of the K9. This confirms that the nmAb-KT epitope lies within K9 peptide P6 that could also be part of the active sites of the K9 killer cytotoxic function. Among the residues of the P6 (³⁹TGGSTDGKQG⁴⁸), it is proposed the epitope for nmAb-KT to be at the position ⁴¹GSTDGK⁴⁶ in K9 Killer toxin. And this 6 residues protrude from the surface of the K9 3D protein molecule model which is imaged by the RasMOL software (Selvakumar, Zhang, et al. 2006).

By using an alanine-scanning method, it has been shown that histidine-35 is essential for the yeast killing activity of K9 (Miyamoto et al. 2005). The DEPC modification experiment which modified the sole histidine residue in K9 showed that loss of this sole histidine residue causes the loss of 70% of the yeast killing activity. Histidine-35 is important for the expression of the killing activity of HM-1, as was shown by using chemical modification and site-directed mutagenesis. Subcellular localization and co-immunoprecipitation experiments indicated that the role of histidine-35 in HM-1 is related to the binding process of K9 to its putative receptor protein, not to the inhibition of 1,3-β- glucan synthase activity (Miyamoto et al. 2006).

1.5.2 The physicochemical characteristics of killer toxin K9

K9 is stable over a wide range of pH which is from 2 to 11 and it is resistant to heat treatment at 100°C for 10 min (Yamamoto et al. 1988a). The isoelectric point (PI) is at pH

9.1. K9 killer toxin can be inactivated by reducing agents, and its S-S bonds are essential for biological activity.

1.5.3 The effect of K9 toxin cytotoxic function

K9 exerts a strong cytotoxic effect on some sensitive yeast, like and some *Candida* genus, which were inhibited by the toxin at concentration as low as 6 µg/ml. However some species are not sensitive and need 100 µg/ml or more toxins to be inhibited, like *Candida albicans*. (Yamamoto et al. 1988b) The minimum inhibitory concentration (MIC) of K9 killer toxin to *Saccharomyces cerevisiae* was 0.4-1.6 µg/ml (S Kasahara et al. 1994; Teturo Yamamoto et al. 1986). The IC₅₀ of K9 for the yeast *Saccharomyces cerevisiae* strain A451 is 0.2µg/ml at the optimal condition. And this killer effect is stronger than aculeacin A and papulacandin B which are the most effective antifungal agents previously known (Komiyama et al. 1996). And the IC₅₀ of the Killer toxin to *Saccharomyces cerevisiae* wild-type BY4743 is 152ng/ml(Miyamoto et al. 2012). But the mammalian cells like mouse L-929 and human HeLa cells are totally unaffected by K9.

1.5.3.1 The condition that affects the K9 cytotoxic activity

The optimum incubation temperature of the K9 cytotoxic effect was 30°C. After 40 min incubation, about 90% cells were killed at the optimal temperature, a condition that make cells at budding index as high as 80%-90%. At 20°C and 37°C, the K9 cytotoxic effect just follows the optimal temperature 30°C in order. The number of dead cells increases with time at 30 °C, 20 °C and 37 °C. However incubation at 0°C, the K9 killer toxin only exerts a limited cytotoxic effect. And cells incubated with K9 at 0°C and then cultured at 30°C after dilution do not die. So the cytotoxic function of K9 is growth-dependent (Komiyama et al. 1996).

1.5.3.2 K9 influences β-1, 3-glucan synthesis

First it is reported that K9 inhibits the β-(1,3)-glucan synthase to exert the cytotoxic function to the yeast. But the K9 killer toxin can not inhibit the synthesis of protein, chitin and mannan (Tetsuro Yamamoto et al. 1986; Teturo Yamamoto et al. 1986).

The overexpression of *HKRI* gene in the yeast can endow the yeast cell with a resistance to K9 killer toxin and can increase the β -glucan contents without affecting the β -glucan synthase activity. And the additional β -1, 3-glucan can protect the yeast from the cytotoxic effect of K9 killer toxin (S Kasahara et al. 1994; Kasahara et al. 1994). K9 killer toxin can inhibit β -1, 3-glucan synthesis detected by the membrane fraction experiments (Teturo Yamamoto et al. 1986). And the inhibitory activity of K9 killer toxin for the purified β -1, 3-glucan synthase was tested by a series of studies. And the IC_{50} value of K9 killer toxin for the membrane bound β -1, 3-glucan synthase activity in the membrane prepared from protoplast was about 50 μ g/ml (Teturo Yamamoto et al. 1986). The IC_{50} of K9 to β -1,3-glucan synthase depends the purification level of the synthase. The IC_{50} of K9 for the purified glucan synthase is 0.5 μ g/ml.

The kinetics of purified β -1, 3-glucan synthase inhibited by K9 was biphasic and the inhibition level of synthase activity by K9 stays at the 50%. It is proposed that the purified glucan synthase is a mixture of two enzymes, one of which is sensitive to the K9 and one other of which is resistant. Or the part of enzymes that is resistant to K9 loses the sites of interaction to K9 (T Takasuka et al. 1995). So the investigation of cellular targets for the K9 is still necessary.

But screening results did not show the highly resistant K9 mutants to participate in β -1, 3-glucan synthesis. K9 is believed to target the Fks1 and Fks2 as caspofungin. And the *FKS1* and *FKS2* deletion mutants showed different levels of sensitivity to K9 also as caspofungin. Conversely *fks1* is K9 sensitive but *fks2* showed mild K9 resistant phenotype (Miyamoto et al. 2011).

1.5.3.3 The morphological changes of the K9 treated cells

1.5.3.3.1 The Pore formation on proliferating yeast

The leakage of cellular components of the yeast treated by K9 is evidenced by the microscopic observation. And the changes of medium were measured by the UV absorption during the K9 incubation. The cell leakage of 50%-60% of the total cells treated by K9 is observed by phase-contrast light microscope. And the scanning electron microscopic analysis show the discharging region is on the budding area and especially on budding growing end site. These results all suggest the K9 generates pores in *Saccharomyces cerevisiae* cell walls.

The bud scars which are sites of previous budding can not be damaged again (Komiyama et al. 1996). And the extra osmotic pressure offered by the osmotic stabilizer sodium chloride (0.15M) can reduce the cytotoxic effect of K9 (Komiyama et al. 1996).

1.5.3.3.2 The yeast spheroplast treated by the K9 killer toxin

The yeast spheroplast without the cell wall can grow large and round after the treatment of the K9 killer toxin. And this morphology change depends on the concentration of the K9 killer toxin. The 50% effective dose of K9 killer toxin is approximately is 2.2×10^{-8} M that agrees well with the K9 IC_{50} value 2.1×10^{-8} M of the sensitive yeast cells. And these spheroplasts treated by K9 killer toxin are round and sufficiently stable to survive several days. So without cell wall, the yeast are not sensitive the K9 killer toxin. But without the treatment of the K9 killer toxin, the normal spheroplast exerts the aberrant morphologies. It is proposed that the cellular β -1,3- glucan synthase as a K9 receptor interacts with K9 and the synthesis of glucan inhibited by K9 results in the perfect round spheroplasts with a smooth surface. In contrast, the glucan synthesis continues in the membrane of the normal spheroplasts without K9 treatment, giving rise to rough surfaces and resulting in the aberrant morphologies (Komiyama et al. 2002).

Experiments of actin staining with Rhodamine Phalloidin show that actin is distributed in the K9 treated spheroplasts cells and no locally concentrated as actin patches. But the strong actin patches were observed in the aberrant morphogenesis spheroplasts cells without the K9 treatment. It suggested the actin can be involved in these morphological changes and regulate the cell morphology by Rho1 protein that is a regulatory subunit of β -1, 3- glucan synthase. And the K9 may inhibit the Rho1 and actin polymerization through the interaction with β -1, 3- glucan synthase (Komiyama et al. 2002).

1.5.3.4 K9 resistant mutants involved in different activities in the *S. cerevisiae*

To search the mechanism of the cytotoxic function for the K9 killer toxin, there are some researches focused on the gene deletion-mutants, which have been found to be resistant or more sensitive to the K9 killer toxin. Masahiko found 8 gene-deletion mutants (six of these eight mutants are newly found) are much more resistant than the wild type through the set of a mutants individually deleted for 4720 non-essential genes. These 8 genes are *ALG3*, *CAX4*, *MNS1*, *OST6*, *STT3*, *FPS1*, *ZUO1* and *YBL083C*. But the other 3 mutants (*hkr1*, *kre6*

and *knr4 /smi1*) which are reported K9 resistant have the mild resistance by IC₅₀ value test (Miyamoto et al. 2011). All these genes involved in the K9 resistance are individually related to the N-glycan formation and maturation, cell wall synthesis and signalling pathways.

1.5.3.4.1 The site of the K9 Binding

Miyamoto postulated that K9 has a two-step action on cell wall-disturbing mechanism. First step is binding and insertion to cell wall structures, and second step is inhibition of β -1, 3-glucan synthase. The first step is more important than the second step (Miyamoto et al. 2012). It is determined that K9 can bind to the yeast subcellular fraction and it is assumed the presence of a glycoprotein has a K9 binding ability in the yeast plasma membrane (Miyamoto et al. 2006). By the IC₅₀ measurement and K9 binding assay results of P22 fractions of *alg3* and *msn1* it suggested that the presence of a sixth mannose residue with no extra mannose link is essential for interaction with K9 binding protein and the yeast-killing action of K9. The P22 fraction that contains the putative K9 binding protein of the plasma membrane had less K9 binding ability in the *alg3* and *msn1* mutant. *ALG3* gene which encodes 1,3-alpha-mannosyltransferase and *MSN1* which encodes 1,2-alpha-mannosidase are related to *CAX4*, *OST6* and *YBL083C* other genes. So the presence of the terminal 1,3-alpha-linked mannose residue of the B-chain of the N-glycan structure is essential for interaction with K9 (Miyamoto et al. 2011).

2.5.3.4.2 HOG and CWI MAP kinase pathways are involved in the response to K9

Some genes have been found maybe involved in the maturation of the K9 receptor that is believed to be a mannoprotein in the plasma membrane. (Kimura et al. 1999; Miyamoto et al. 2006). And the *fps1* mutants are strongly resistant to K9 and *hog1* and *slt2* cells showed more K9 susceptibility than the wild type (Miyamoto et al. 2012). Fps1 protein is an aquaglycerol channel protein associated with regulation of turgor of *Saccharomyces cerevisiae*. Fps1 channel can open and export the intracellular glycerol to outside in a low osmotic pressure and close in a high osmotic pressure environment. In contrast, some yeast strains that are K9 resistant miss Fps1 protein, like *C.albicans* (Yamamoto et al. 1988b). Fps1 is reported to work cooperatively with HOG pathway. And both the HOG and CWI MAP kinase pathways are involved in the response to K9 action (Figure 9). In the wild type, both Slt2 and Hog1 that has an opposite role to Fps1 are diphosphorylated in response to the K9. It is reported that the dihospho-Hog1 is important for the induction of diphospho-Slt2, but not in opposite, in

response to the K9 killer toxin. So it is suggested that HOG and CWI pathways are activated sequentially in response to the K9 killer toxin. And the Ssk1 branch is involved in response to K9, rather than the Sho1 branch in the HOG pathway. It is postulated that activation of both the HOG and CWI pathways will activate glucan synthase.

Knr4 protein, whose absence also leads to K9 resistance, is also involved in the CWI MAP kinase pathway. (See 1.4 *KNR4*)

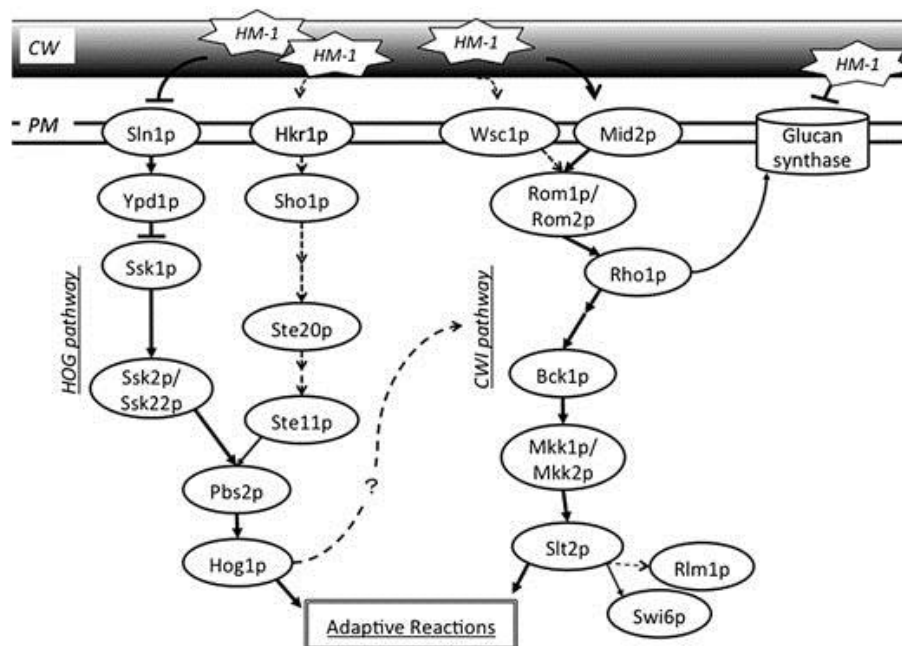


Figure 9: K9/HM-1 activates both HOG and CWI pathways of *S. cerevisiae* to induce adaptive reactions.

The components of the HOG and CWI pathways involved in the adaptive response to HM-1 are shown in the model. The components not mentioned in the Discussion are omitted (Miyamoto et al. 2012).

1.6 AFM-Tools for investing the yeast cell wall

Created in 1986 (Binnig et al. 1986), Atomic Force Microscopy (AFM) allows scanning a sample in air or liquid, making possible its use on living cells in their physiological conditions. Therefore AFM is a useful technology to evaluate the effects of various stresses on the cell wall of living yeast cells (Francois et al. 2013) such as thermal stress (Pillet et al. 2014), or antifungal drug treatment (Formosa et al. 2013).

Moreover imaging the cell surface at the nanometre resolution directly on living cells, AFM allows recording force measurement between the cell surface and the AFM tip. Therefore AFM has emerged as a cutting-edge technology to study the biophysical properties of the cell wall of living cells, under aqueous controlled conditions (Dufrêne 2004; Dufrêne 2008).

1.6.1 Main parts of AFM

The AFM is constituted mainly by the cantilever, the sharp tip, the sample stage and the optical deflection system. The optical deflection system is constituted by a laser and a photodetector that is also called photodiode (Figure 10 A). There is a laser beam can recite on the back of the tip of the cantilever and the photodectctor can recorde and measure the difference of the position and the voltage. And finally the photodetector gives a direct measurement of the vertical deflection (deviation) of the cantilever. The AFM can generate a tridimensional image of the sample by scanning the tip over the surface, while the force applied on the surface is maintained constant by a piezoelectric scanner, which allows by applying correct voltage a three dimensional positioning. What is more, force *versus* distance curves can be recorded between the tip and the surface with piconewton sensitivity. When the tip is approached to the surface sample and then retracted in the Z direction, forces versus distance (F-D) curves are recorded. On biological samples, the F-D curve consists in a non-contact and a deformation component (A review Francois et al. 2013).

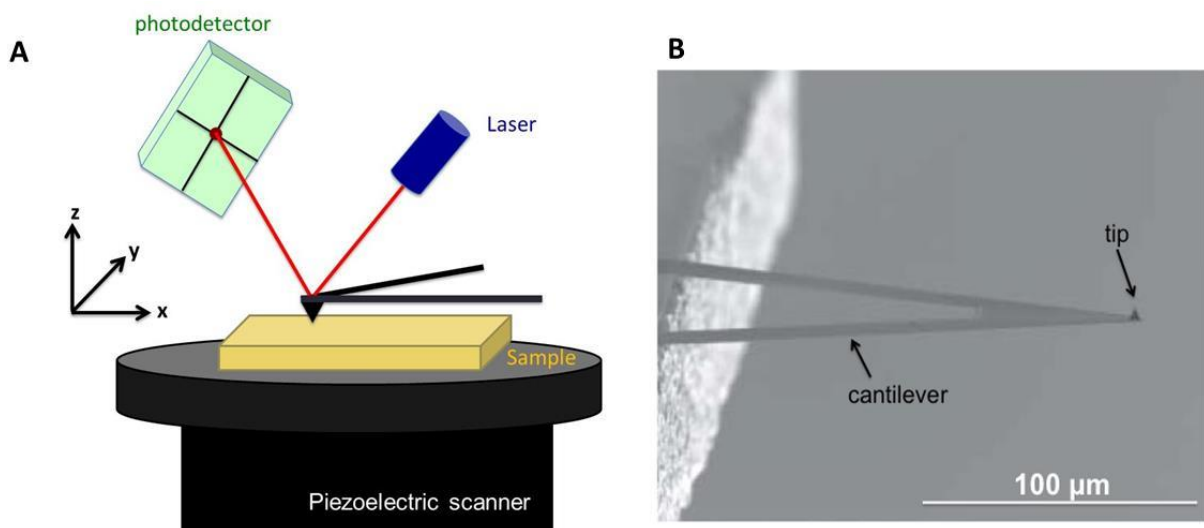


Figure 10: A scheme of an atomic force microscope (A) and MEB image of the AFM-cantilever with at this end a tip (B).

1.6.2 Immobilizing the yeast cells

It is required to be fixed for the samples when the AFM scan the samples. There are several methods applied to the yeast cells for immobilizing: (1) air drying and chemical fixation of a cell suspension on glass slide; (2) trapping yeast cells on soft material such agar or gelatin and (3) mechanical trapping in porous polycarbonate membranes. Now there is an alternative method for trapping yeast cells in micrometre-size chambers, produced by soft lithography technology and using an elastomer such as polydimethylsulfoxane (PDMS) (Figure 11. For a review Francois et al. 2013). The advantage of the last method is its efficiency to trap numbers of cells of different sizes while keeping the viability and integrity of the cells.

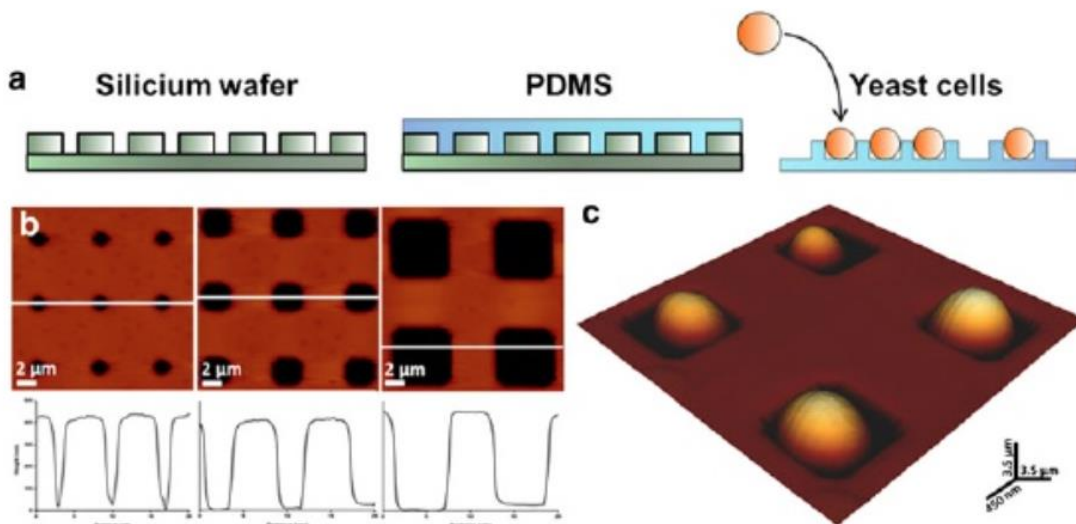


Figure 11: Immobilization of living *Saccharomyces cerevisiae* cells.

A Schematic representation of yeast cells in PDMS microstructured stamps. B Height AFM images and corresponding cross-sections of microstructured PDMS stamps. C *S. cerevisiae* cells trapped in the microstructured PDMS stamps as imaged by atomic force microscopy (Francois et al. 2013)

1.6.3 Imaging mode

Two modes can be used to image yeast cells, which are contact mode and tapping mode. For the contact mode, the tip is continuously in contact with the surface of the sample and scans this surface horizontally with a constant force (Figure 12a). For the tapping mode, the cantilever is oscillating near its resonance frequency. The tip oscillates until to get contact with the surface and the modification of the amplitude of oscillation allows measuring the topography (Figure 12b). Avoiding to damage the cell surface in the tapping contact, the tip

has an intermittent contact with the sample, lateral forces between the tip and the surface are reduced (Alsteens et al. 2012). The QI mode (QI for Quantitative Imaging) was developed recently by JPK Instruments and can collect the force curves at the same speed and resolution for normal imaging. In QI mode the tip can move from point to point across the surface and the AFM tip is completely detached from the surface before moving to the next point (Chopinnet et al. 2013).

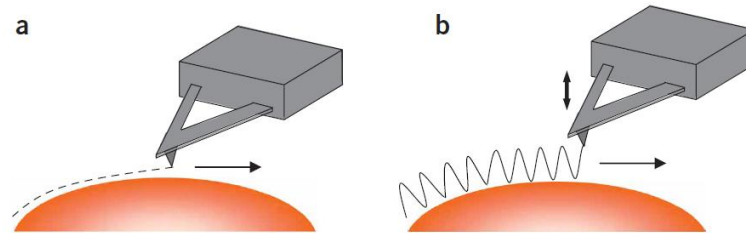


Figure 12: Contact mode. (a) and tapping mode (b)
(Dufrêne, 2008, Nature Protocol)

Imaging modes are very useful to collect tridimensional images of yeast cells to determine structural elements on the surface of the yeast cell such as the birth and bud scars (Dague et al. 2010), but also to investigate the morphological modification upon different stresses (references).

Except imaging, the AFM is also operated in the force spectroscopy mode as a force machine able to record force measurements between the tip and the surface.

1.6.4. Force spectroscopy

In the force spectroscopy mode, the tip is continuously approached and retracted from the surface and the deflection of the cantilever is monitored in function of the vertical position of the piezoelectric scanner. Actually the voltage on the photodetector is measured in function of the vertical piezo displacement and this voltage (I in Volts) is converted into a deflection (d) which have the dimension of a length according to: $d = S \cdot I$ where S is the sensibility of the cantilever (in $\text{m} \cdot \text{V}^{-1}$). Using the spring constant of the cantilever (k_{cant} in $\text{N} \cdot \text{m}^{-1}$), this deflection can be converted in force (F) using the Hooke's law: $F = k_{\text{cant}} \cdot d$.

The force-distance curves provide numbers of information. When the tip is in contact with the sample, the sample 'resists' and there is a deformation of both, the cantilever and the sample. This part of the curve give us access to nanomechanicals properties of the yeasts such as cellular spring constant, elasticity modulus or Young modulus (Mercadé-Prieto et al., 2013; Pelling et al., 2004). When the tip is moved away from the sample, one hysteresis can be recorded, resulting in the adhesion of the tip with the sample. This tip-surface interaction is dependent of the time of contact of the tip, the area of the surface and the surface energy.

Chapter 2 Material and Methods

2.1 Strains, plasmid and primers

2.1.1 Strains

Table 1: Strain list

Yeast strains	Genotype	Reference
BY4741	<i>MAT a; his3Δ1 leu2Δ0; met15Δ0; ura3Δ0</i>	Brachmann <i>et al.</i> 1998
PJ69-4a	<i>MAT a trp1-901 leu2-3,112 ura3-52 his3-200 gal4Δ GAL80d LYS2::GAL1-HIS3 GAL2-ADE2 met2:: GAL7-lacZ</i>	James <i>et al.</i> 1996
<i>H.marakii</i> (IFO0895)		Tetsuro Yamamoto <i>et al.</i> 1986
BYG53	<i>MAT a; his3Δ1 leu2Δ0; met15Δ0; ura3Δ0; KNR4::KNR4-GFP-URA</i>	This study
BEM1 G53	BY4741; Mata; <i>his3Δ1 leu2Δ0; met15Δ0; ura3Δ0; YBR200W::kanMX4; KNR4::KNR4-GFP-URA</i>	This study
BEM2G53	BY4741; Mata; <i>his3Δ1 leu2Δ0; met15Δ0; ura3Δ0; YER155C::kanMX4; KNR4::KNR4-GFP-URA</i>	This study
BN11 G53	BY4741; Mata; <i>his3Δ1 leu2Δ0; met15Δ0; ura3Δ0; YNL271C::kanMX4; KNR4::KNR4-GFP-URA</i>	This study
BUD6 G53	BY4741; Mata; <i>his3Δ1 leu2Δ0; met15Δ0; ura3Δ0; YLR319C::kanMX4; KNR4::KNR4-GFP-URA</i>	This study
PCL1 G53	BY4741; Mat a; <i>his3Δ1 leu2Δ0; met15Δ0; ura3Δ0; YNL289w::kanMX4; KNR4::KNR4-GFP-URA</i>	This study
PCL2 G53	BY4741; Mat a; <i>his3Δ1 leu2Δ0; met15Δ0; ura3Δ0; YDL127w::kanMX4; KNR4::KNR4-GFP-URA</i>	This study
RRD1G53	BY4741 ; Mat a ; <i>his3Δ1 leu2Δ0; met15Δ0; ura3Δ0 ; YIL153w ::kanMX4; KNR4::KNR4-GFP-URA</i>	This study
SPA2 G53	BY4741 ; Mat a ; <i>his3Δ1 leu2Δ0; met15Δ0; ura3Δ0 ; YLL021W ::kanMX4; KNR4::KNR4-GFP-URA</i>	This study
YCK1 G53	BY4741 ; Mat a ; <i>his3Δ1 leu2Δ0; met15Δ0; ura3Δ0 ; YHR135C ::kanMX4; KNR4::KNR4-GFP-URA</i>	This study
TPD3G53	BY4741; Mat a; <i>his3Δ1 leu2Δ0; met15Δ0; ura3Δ0; YAL016W::kanMX4; KNR4::KNR4-GFP-URA</i>	This study
<i>knr4</i> ^{S200AS203A}	BY4741; <i>KNR4</i> ^{S200A203A} - terminator ::kanMX4	This study
<i>knr4</i> ^{S200DS203D}	BY4741; <i>KNR4</i> ^{S200D203D} - terminator ::kanMX4	This study
<i>knr4ΔN</i>	BY4741; <i>KNR4Δ1-80</i> - terminator ::kanMX4	This study

<i>knr4ΔN+C</i>		BY4741; <i>KNR4ΔI-80, Δ340-505-terminator::kanMX4</i>	This study
<i>knr4ΔC</i>		BY4741; <i>KNR4Δ340-505-terminator::kanMX4</i>	This study
Mutants haploids			
Mutants	Gene System name		
<i>ace2</i>	YLR131C	BY4741 ; Mata ; his3Δ1 leu2Δ0; met15Δ0; ura3Δ0 ; YLR131C ::kanMX4	Yeast Knock Out collection (YKO)
<i>bem1</i>	YBR200W	BY4741 ; Mata ; his3Δ1 leu2Δ0; met15Δ0; ura3Δ0 ; YBR200W ::kanMX4	YKO
<i>bem2</i>	YER155C	BY4741 ; Mata ; his3Δ1 leu2Δ0; met15Δ0; ura3Δ0 ; YER155C ::kanMX4	YKO
<i>bni1</i>	YNL271C	BY4741 ; Mata ; his3Δ1 leu2Δ0; met15Δ0; ura3Δ0 ; YNL271C ::kanMX4	YKO
<i>bnr1</i>	YIL159W	BY4741 ; Mata ; his3Δ1 leu2Δ0; met15Δ0; ura3Δ0 ; YIL159W ::kanMX4	YKO
<i>bud6</i>	YLR319C	BY4741 ; Mata ; his3Δ1 leu2Δ0; met15Δ0; ura3Δ0 ; YLR319C ::kanMX4	YKO
<i>elm1</i>	YKL048C	BY4741 ; Mata ; his3Δ1 leu2Δ0; met15Δ0; ura3Δ0 ; YKL048C ::kanMX4	YKO
<i>hbt1</i>	YDL223C	BY4741 ; Mata ; his3Δ1 leu2Δ0; met15Δ0; ura3Δ0 ; YDL223C ::kanMX4	YKO
<i>hsl1</i>	YKL101W	BY4741 ; Mata ; his3Δ1 leu2Δ0; met15Δ0; ura3Δ0 ; YKL101W ::kanMX4	YKO
<i>knr4</i>	YGR229C	BY4741 ; Mata ; his3Δ1 leu2Δ0; met15Δ0; ura3Δ0 ; YGR229C ::kanMX4	YKO
<i>pam1</i>	YDR251W	BY4741 ; Mata ; his3Δ1 leu2Δ0; met15Δ0; ura3Δ0 ; YBR251w ::kanMX4	YKO
<i>pea2</i>	YER149C	BY4741 ; Mata ; his3Δ1 leu2Δ0; met15Δ0; ura3Δ0 ; YER149C ::kanMX4	YKO
<i>pcl1</i>	YNL289W	BY4741; Mat a; his3Δ1 leu2Δ0; met15Δ0; ura3Δ0; YNL289w::kanMX4	YKO
<i>pcl2</i>	YDL127W	BY4741; Mat a; his3Δ1 leu2Δ0; met15Δ0; ura3Δ0; YDL127w::kanMX4	YKO
<i>rrd1</i>	YIL153W	BY4741 ; Mat a ; his3Δ1 leu2Δ0; met15Δ0; ura3Δ0 ; YIL153w ::kanMX4	YKO
<i>spa2</i>	YLL021W	BY4741 ; Mat a ; his3Δ1 leu2Δ0; met15Δ0; ura3Δ0 ; YLL021W ::kanMX4	YKO
<i>sbe2</i>	YDR351W	BY4741 ; Mat a ; his3Δ1 leu2Δ0; met15Δ0; ura3Δ0 ; YDR351W ::kanMX4	YKO
<i>sbe22</i>	YHR103W	BY4741 ; Mat a ; his3Δ1 leu2Δ0; met15Δ0; ura3Δ0 ; YHR103W ::kanMX4	YKO
<i>tpd3</i>	YAL016W	BY4741; Mat a; his3Δ1 leu2Δ0; met15Δ0; ura3Δ0; YAL016W::kanMX4	YKO
<i>yck1</i>	YHR135C	BY4741 ; Mat a ; his3Δ1 leu2Δ0; met15Δ0; ura3Δ0 ; YHR135C ::kanMX4	YKO
<i>yck2</i>	YNL154C	BY4741 ; Mat a ; his3Δ1 leu2Δ0; met15Δ0; ura3Δ0 ; YNL154C ::kanMX4	YKO
<i>cna1</i>	YLR433C	BY4741 ; Mat a ; his3Δ1 leu2Δ0; met15Δ0; ura3Δ0 ; YLR433C ::kanMX4	YKO
<i>slt2</i>	YHR030C	BY4741 ; Mat a ; his3Δ1 leu2Δ0; met15Δ0; ura3Δ0 ; YHR030C ::kanMX4	YKO
<i>pph21</i>	YDL134C	BY4741 ; Mat a ; his3Δ1 leu2Δ0; met15Δ0; ura3Δ0 ; YDL134C ::kanMX4	YKO

<i>pph22</i>	YDL188C	BY4741 ; Mat a ; his3Δ1 leu2Δ0; met15Δ0; ura3Δ0 ; YDL188C ::kanMX4	YKO
<i>cdc55</i>	YGL190C	BY4741 ; Mat a ; his3Δ1 leu2Δ0; met15Δ0; ura3Δ0 ; YGL190C ::kanMX4	YKO
Bacteria strains		Genotype	Reference
DH5α		Sup E44 Δlac U169(Δ80 lacZ Δ M15) hsdR7 recA1 endA1 gyrA96 thi-1 rel A1	Invitrogen

Construction of the yeast strains

Genome integration in mutants:

11 strains BYG53, BEM1G53, BEM2G53, BNI1G53, BUD6G53, PCL1G53, PCL2G53, RRD1G53, SPA2G53, YCK1G53, TPD3G53 were constructed by genome integration of a Knr4-GFP gene fusion at the endogenous *KNR4* gene locus, into the strains BY4741 (control strain) and the following deletion mutants from the YKO collection (Open Biosystems) *bem1*, *bem2*, *bni1*, *bud6*, *pcl1*, *pcl2*, *rrd1*, *spa2*, *yck1*, *tpd3*. Transforming DNA fragment was obtained by AgeI digestion fragment from plasmid pHM53. There are 2 AgeI digestion sites in this plasmid. We digested pHM53 with AgeI enzyme and kept the biggest fragment which contains *KNR4-GFP* and *URA3* gene. Integration into the Yeast genome was performed by lithium acetate transformation and selection on solid media lacking uracile. The details of the integration are shown in Figure 13.

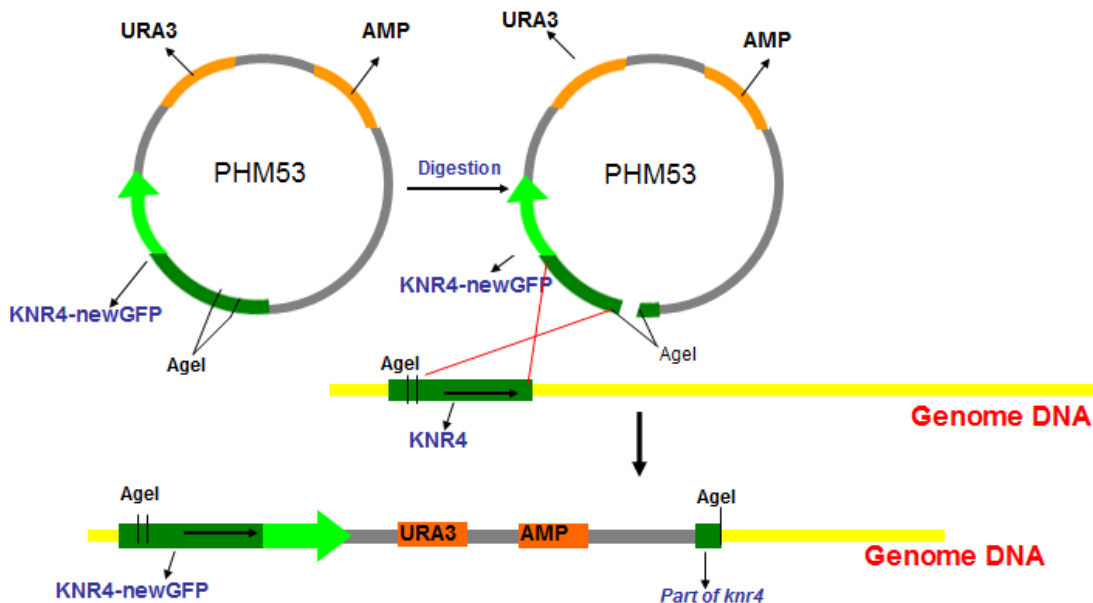


Figure 13: Integration of the KNR4-newGFP into the Genome DNA

5 mutants *knr4*^{S200AS203A}, *knr4*^{S200DS203D}, *knr4*Δ*N*, *knr4*Δ*N*+*C* and *knr4*Δ*C* were constructed by genome integration of mutant alleles *knr4* S200AS203A, S200DS203D and truncated fragments *KNR4* (80-550), *KNR4* (80-340) and *KNR4* (1-340) respectively. Transforming DNA fragments were obtained by EagI-KpnI digestion of plasmid PRS426-*KNR4*^{S200D203D}-T-KAN, PRS426-*KNR4*^{S200A203A}-T-KAN, PRS426-*KNR4*Δ*N*-T-KAN, PRS426-*KNR4*Δ*N*+*C*-T-KAN, PRS426-*KNR4*Δ*C*-T-KAN.

2.1.2 Plasmids

Table 2: Plasmids list

Plasmid		
pGEM-T-KNR4	<i>KNR4</i> gene in pGEM-T	F. Durand Thesis
PGEMT-terminator XhoI-Sal1/Kpn1	<i>KNR4</i> gene with terminator(XhoI-Sal1/Kpn1) in pGEMT	F. Durand Thesis
PGEMT-Knr4 ^{S200D203D}	<i>KNR4</i> (598-600,607-609) site mutagenesis from serin to Glutamic acid in pGEM-T	This study
PGAD424	Gal4-activating domain containing plasmid for two-hybrid system	Amersham
PGAD424-TYS1	<i>TYS1</i> ORF fused to Gal4 activating domain in pOBD80	Dagkessamanskaia <i>et al.</i> 2001
PGAD424-CNA1	<i>CNA1</i> ORF fused to Gal4 activating domain in pOBD80	
POBD80	Gal4 binding domain-containing plasmid for two-hybrid systme	Louvet <i>et al.</i> 1997
pOBD80-KNR4	<i>KNR4</i> ORF fused to Gal4 binding domain in pOBD80	Dagkessamanskaia <i>et al.</i> 2001
pOBD80-KNR4 ^{S200A203A}	<i>KNR4</i> ^{S200A203A} mutant fused to Gal4 binding domain in pOBD80	H. Martin-Yken
pOBD80-KNR4 ^{S200D203D}	<i>KNR4</i> ^{S200D203D} mutants fused to Gal4 binding domain in pOBD80	This study
pRS426-Knr4	<i>KNR4</i> Gene with promoter in pRS426 which with <i>URA3</i> marker	F. Durand Thesis
pFD19	<i>KNR4</i> (1-340) Gene in PRS426 which with <i>URA3</i> marker	F. Durand Thesis
pFD20	<i>KNR4</i> (80-340) Gene in pRS426 which with <i>URA3</i> marker	F. Durand Thesis
pFD32	<i>KNR4</i> (1-80) Gene in pRS426 which with <i>URA3</i> marker	F. Durand Thesis
pFD33	<i>KNR4</i> (80-505) Gene in pRS426 which with <i>URA3</i> marker	F. Durand Thesis
pRS426	Vector contains the marker <i>URA 3</i>	Sikorski and Hieter, 1989
pRS426-Knr4 ^{S200D203D}	<i>KNR4</i> ^{S200D203D} with promoter	This study

	mutants in PRS426	
pRS426-Knr4 ^{S200A203A}	KNR4 ^{S200A203A} with promoter mutants in PRS426	This study
pRS316	Centromeric vector with URA3 marker	Sikorski and Hieter, 1989
pHM38	KNR4 with promoter cloned in PRS316	Martin <i>et al.</i> 1999
pFB1	KNR4 ^{S200A203A} with promoter in PRS316	Basmaji <i>et al.</i> 2006
pRS316- KNR4 ^{S200D203D}	KNR4 ^{S200D203D} with promoter (Between 2 PvuI sites from PRS426-Knr4 ^{S200D203D}) in PRS316	This study
pRS315	Centromeric vector with <i>LEU2</i> marker	Sikorski and Hieter, 1989
pRS315-KNR4	KNR4 with promoter cloned in PRS315	Dagkessamanskaia <i>et al.</i> 2010
pRS315- KNR4 ^{S200A203A}	KNR4 ^{S200A203A} with promoter (Between 2 PvuI sites from PFB1)	This study
pRS315- Knr4 ^{S200D203D}	KNR4 ^{S200D203D} with promoter (Between 2 PvuI sites from PRS426-Knr4 ^{S200D203D}) in PRS316	This study
pHM43	KNR4 fused GFP in PUG35	Dagkessamanskaia <i>et al.</i> 2010
pUG35-KNR4 ^{S200D203D}	KNR4 ^{S200D203D} fused GFP in PUG35	This study
pHM53	KNR4 fused to GFP, with its own promoter, in the integrative vector pRS306	H. Martin Thesis
pGEMT-KNR4-T-Kan	<i>KNR4</i> gene with its terminator with KanMX4 in pGEM-T (Promega)	This study
pRS426-KNR4-T-Kan	<i>KNR4</i> gene with its promoter and terminator::KanMX4 in PRS426	This study
PRS426-KNR4 ^{S200D203D} -T::KanMX4	<i>KNR4</i> ^{S200D203D} with its promoter and terminator::KanMX4 in PRS426	This study
PRS426-KNR4 ^{S200A203A} -T::KanMX4	<i>KNR4</i> ^{S200A203A} with its promoter and terminator::KanMX4 in PRS426	This study
pRS426-KNR4 Δ N-T::KanMX4	<i>KNR4</i> Δ N(80-505)+ promoter and terminator::KanMX4 in PRS426	This study
PRS426-KNR4 Δ N+C-T::KanMX4	<i>KNR4</i> Δ N+C(80-340)+ prom. and terminator::KanMX4 in PRS426	This study
PRS426-KNR4 Δ C-T::KanMX4	<i>KNR4</i> Δ C (1-340)+promoter and terminator::KanMX4 in PRS426	This study
PSG2	PLG669 (vector Amp, <i>URA 3</i> ; fusion with <i>pCYC1-LacZ</i>) with 6WCE-lacZ	Samuel GELIS
PAL 3	<i>pFKS2</i> (Δ wce) -lacZ	Arnaud LAGORCE thesis

pAL5	<i>pFKS2 (Awce)₆-lacZ</i>	Arnaud LAGORCE thesis
YIL117C-LacZ	Duplicated binding site from YLC117C fused to the minimal <i>CYCI</i> promoter	Jung et al. 2002
PNV7-mkk1 ³⁸⁶	Mkk1 ³⁸⁶ with GAL 1 promoter	Watanabe et al. 1995

Construction of the plasmids

PGEMT-Knr4^{S200D203D}

We designed the primers for mutagenesis to replace Serin residues 200 and 203 by Glutamic acid residues (D), and on this pair of primers we also removed the BglII digestion site of *Knr4* gene, so that it should be absent from the mutant allele *knr4^{S200D203D}*. So it is easy to check the mutant *knr4^{S200D203D}*. And then do the mutagenesis PCR temperature cycling for *knr4^{S200D203D}* taking the PGEMT-*KNR4* as the template. The main steps can be seen in methods. After checking by enzyme digestion and sequencing, we obtained the **PGEMT-*knr4^{S200D203D}*** mutant vector.

pOBD80-KNR4^{S200D203D}

pOBD80-*KNR4^{S200D203D}* contains *KNR4^{S200A203A}* mutants fused to Gal4 binding domain in pOBD80. The first plasmid pOBD80-*KNR4^{S200D203D}* (PstI-BamHI) is obtained by the ligation of the big fragment of pOBD80 plasmid and *KNR4^{S200A203A}* fragments from pGEMT-*knr4^{S200D203D}* that are digested by the PstI-BamHI. And then digest pOBD80-*KNR4^{S200D203D}* (PstI-BamHI) by NcoI, so the BamHI site has been cut for the expression.

pRS426-Knr4^{S200D203D}

pRS426-Knr4^{S200D203D} is obtained by ligating the fragment Knr4^{S200D203D} that is amplified by PCR from pGEMT-*knr4^{S200D203D}* by a pair of primers BamHI-Knr4-start(ATG included) and Knr4-505AntisensXhoI and the pRS426 digested by BamHI-XhoI.

pRS426-Knr4^{S200A203A}

pRS426-Knr4^{S200A203A} is obtained by ligating the fragment Knr4^{S200A203A} that is amplified by PCR from pGEMT-*knr4^{S200D203D}* by a pair of primers BamHI-Knr4-start(ATG inclu) and Knr4-505AntisensXhoI and the pRS426 digested by BamHI-XhoI.

pRS316- KNR4^{S200D203D}

pRS316 plasmid can be divided into two parts 2974bp(*URA3*) and 1913bp by the enzyme PvuI. And the pRS426-Knr4^{S200D203D} is also divided into two parts 4380bp (knr4 with promoter) and 3806bp(*URA3*). pRS316- *KNR4*^{S200D203D} is obtained by the linkage the 2974bp (*URA3*) part of pRS316 plasmid and the 4380bp (*KNR4* with promoter) of pRS426-Knr4^{S200D203D}.

pRS315- Knr4^{S200D203D}

Two parts 4095bp (*URA3*) and 1913bp of the pRS315 are obtained by the digestion of PvuI. And the pRS315- Knr4^{S200D203D} is obtained by by ligating the 4095bp (*URA3*) part of pRS315 plasmid and the 4380bp (*KNR4* with promoter) of pRS426-Knr4^{S200D203D}.

pRS315- KNR4^{S200A203A}

The plasmid FB1 (pRS316- *KNR4*^{S200A203A}) are divided into 5kp (*KNR4* with promoter) and 2974bp (*URA 3*) by the PvuI. To get pRS315-Knr4^{S200D203D} we link the 5kp (*KNR4* with promoter) and the 4095bp (*URA3*) part of pRS315 plasmid.

pGEMT-KNR4-T-Kan

We cut the plasmid pGEMT-terminator *KNR4* XhoI-Sal1/Kpn1 by AppII (in the terminator) and did the Infusion cloning (BD) with gene KAN which is amplified by a pair of primers Kan Start and Kan End from plasmid pFA Kamnx4. So PGEMT-KNR4-T-Kan contains gene BamHI-KNR4-Xho1-Terminator(part)-KAN-terminator(part)-KpnI.

pRS426-KNR4-T-Kan

We cut pRS426-KNR4, which contains the promoter by XhoI-Kpn1 and link with the terminator with KAN (Xho1-Terminator (part)-KAN- terminator (part)-KpnI) of the plasmid pGEMT-KNR4-T-KAN. And the pRS426-KNR4-T-Kan contains the promoter, *KNR4* and terminator with KAN inside.

Plasmids pRS426-KNR4^{S200D203D}-T::KanMX4, pRS426-KNR4^{S200A203A}-T::KanMX4, pRS426-KNR4 Δ N-T::KanMX4, PRS426-KNR4 Δ N+C- T::KanMX4 and PRS426-KNR4 Δ C-T::KanMX4 are obtained by the combination terminator-KAN (Xho1-Terminator(part)-KAN-terminator(part)-KpnI) of the plasmid PGEMT-KNR4-T-KAN) with Xho1-Kpn1 digested plasmids pRS426-KNR4^{S200D203D}, PRS426-KNR4^{S200A203A}, PFD33, PFD20 and PFD1.

2.1.3 Primers

Table 3 Oligos list

Name	Oligo Sequence
Spe1-Knr4-start	CGCGCGACTAGTATGGATCTATTCAAAGAAAAGTT
HindIII-Knr4-end	GCGCGCAAGCTTTAAAGCTATATTTCAAATTCTTC
Amont-prom KNR4	GCAAATTGTCTGACGGCGACGC
Rev GFP Nco1	CTAAGGTTGGCCATGGAAGT
S200DS203D	GAATCGATGACCGGTAATTC
BamHI-Knr4-start(ATG included)	GTAAACGGATCCATGGATCTATTCAAAGAAAAGTT
Knr4-505AntisensXhoI	CTCGAGTCATAAAGCTATATTTCAAATTCTTC
Kan Start	AGGTTTCAACCTTAAGACATGGAGGCCAGAAATAC
Kan End	GTATAAATTTCTTAAGCGACCGACATTCACATACG

2.1.4 Enzymes

All the restriction enzymes are from NEB New England Biolabs

2.1.5 Media and culture conditions

2.1.5.1 For bacteria

LB Medium

- Trypton (1%): 10g
- Yeast extract (0.5%): 5g
- Nacl (0.5%): 5g
- Bacto- agar (2%): 20g (For solid media)
- Water to 1 litre

Sterilize at 121°C for 20min

Media with antibiotics

Ampicillin: A final concentration of Ampicilline (Sigma, no.A9518) 50µg/ml in LB medium was added at temperature below 60°C. Ampicilline was prepared as a 100mg/ml stock in the sterilized water at -20°C.

Kanamycin: Add Kanamycin to LB medium to a final concentration 75µg/ml at temperature below 60°C.

Culture condition for bacteria

Bacteria *Eschrichia coli DH5a* cells were cultivated on solid plate in incubator at 37°C or in liquid media at 37°C with shaking speed of 200rpm/min.

2.1.5.2 Media and condition for yeasts

YPD (YEPE, YP Dextrose)

- Yeast extract (1%): 10g
- Bacto-peptone (2%): 20g
- Glucose (2%): 20g
- Bacto-agar (2%): 20g(For solid media)
- Water to 1 litre

Sterilize at 121°C for 20min

YPD is a complete rich medium for yeast routine growth.

G418 rich medium

Add geneticin (G418) to YPD to reach a final concentration of 200µg/ml when temperature is below 60°C.

ClonNAT rich medium

Add nourseothricin (NAT) to YPD to reach a final concentration of 100µg/ml.

YPD medium for producing K9

The liquid YPD was adjusted to pH4.3 by the addition of 50 mM Na citrate and then sterilised at 121°C for 20min.

YNB glucose (Yeast nitrogen base + Glucose)

- Bacto-yeast nitrogen base w/o ammonium sulphate (0.17%): 1.7 g

- Ammonium sulphate (0.5%): 5g

- Glucose (2%): 20g

- Bacto-agar (2%): 20g

- Water to 1 Litre

Sterilize at 121°C for 20min

YNB glucose medium is a minimal synthetic media for yeasts routine growth.

5'-FOA

0.05% 5'-FOA (0.5g/L) are added to the YNB medium. This medium is used to select for the loss of *URA3* function in *Saccharomyces cerevisiae*.

Sythetic complete (SC) medium

Sythetic complete (SC) medium is a SD medium supplemented with complete drop-out mix.

To obtain SC medium, add the following amount to 1 liter SD medium:

- Complete drop-out mix: 10ml
- Uracile (1%): 4ml
- Adenine (1%): 1ml
- Tyrosine (1%): 5ml

Culture condition for yeasts

Yeast cells were cultivated in liquid medium at 30°C in shaking flasks at a shaking speed of 200rpm/min or in solid agar at 30°C in the incubator.

2.2 Molecular biology methods

2.2.1 PCR

2.2.1.1 PCR by taq DNA polymerase (Biolabs, No. M0267S).

PCR mix:

- Primers: 0.25 μ M final
- dNTP: 0.25mM final
- Buffer: 1 \times final
- Enzyme: Taq DNA polymerase (5U /ml final)
- Plasmid or genomic DNA as template: about 100ng
- Disterilised water to final volume

PCR cycling procedure

Step	Cycle times	Cycle program	Temperture	Time
Step1	1	Initial Denaturation	94°C	5min
Step2	30	Dnaturation	94°C	30S
		Annealing	TM of primer	30S
		Extension	72°C	1 min for 1kb
Step3	1	Final extersion	72°C	7min

2.2.1.2 PCR by fusion DNA polymerase

PCR mix:

- Primers : 0.25µM final
- dNTP : 0.25mM final
- Fusion 5×HF Reation Buffer: 1× final
- Enzyme : fusion DNA polymerase (20U /ml final)
- Plasmid or genomic DNA as template : about 100ng
- Disterilised water to final volume

PCR cycling

Step	Cycle times	Cycle program	Temperture	Time
Step1	1	Initial Denaturation	98°C	30S
Step2	30	Dnaturation	98°C	10S
		Annealing	Tm of primer+3°C	30S
		Extension	72°C	30S for 1kb
Step3	1	Final extersion	72°C	7min

2.2.2 Mutagenesis PCR

Site directed mutagenesis of *KNR4* gene was performed by Recombination PCR. In this study we did mutagenesis of the plasmid pGEMT-Knr4 to the pGEMT-Knr4^{S200D203D}. We designed

mutagenesis primers for to replace the two Serin residues 200 and 203 by Aspartic acid residues (D), and on this pair of primers we also removed the BglIII digestion site of *KNR4* gene, so that it should be absent from the mutant allele *knr4*^{S200D203D}. So it is easy to check the mutant *knr4*^{S200D203D}. We performed the mutagenesis PCR to get *knr4*^{S200D203D} temperature cycling taking the pGEMT-*KNR4* as the template. The main steps of the whole procedure can be seen in the Figure 14. After checking by enzyme digestion and sequencing, we obtained the pGEMT-*knr4*^{S200D203D} mutant vector. About 200ng plasmid template is necessary. Temperature cycling, Phusion DNA polymerase (NEB) and PCR system and are introduced at step 2. For the step3 (digestion), we added 5 µl Buffer NEB4 and 1µl DpnI with PCR products and incubated the mixture at 37°C for 1h, then we added another 1µl DpnI and incubated at 37°C for another 1h. After checking by electrophoresis of 5µl of the product on 1% agarose gel, we transformed the product into the *E. coli* DH5α cells for saving and amplication.

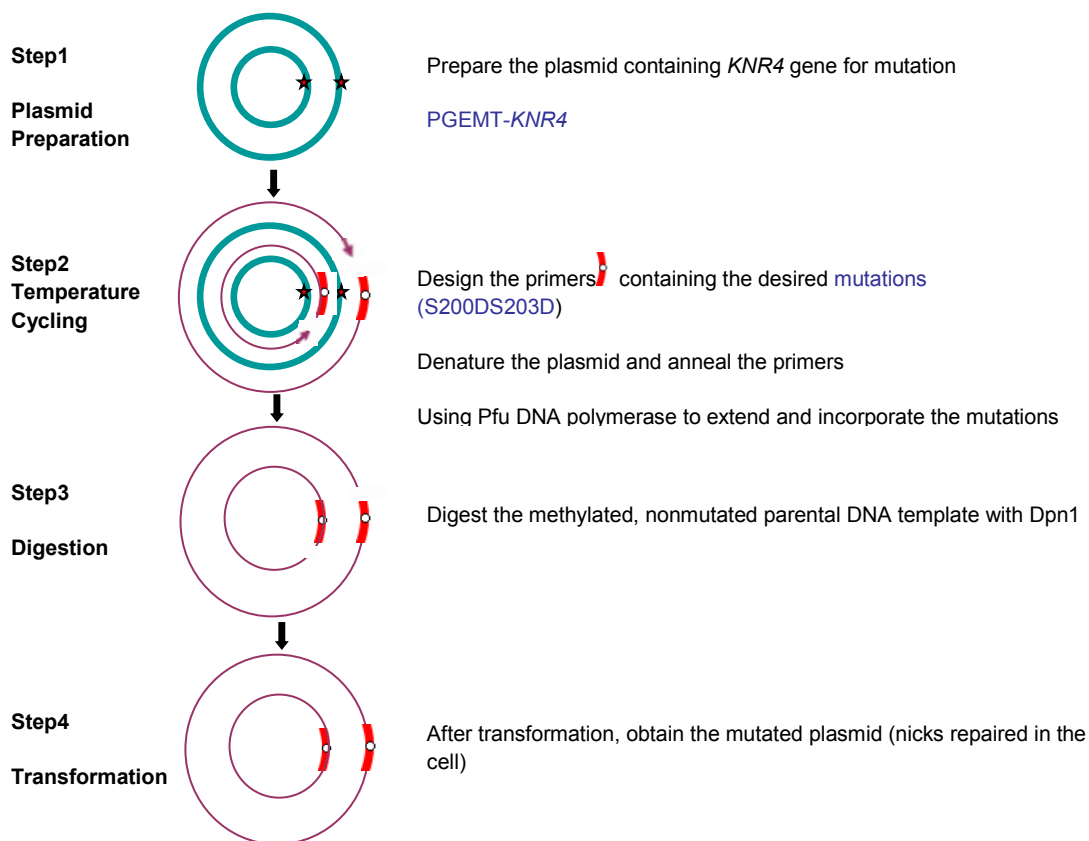


Figure 14: Site-directed Mutagenesis on the plasmid pGEMT-*KNR4* to create pGEMT-*knr4*^{S200D203D}

2.2.3 In-fusion cloning

The In-fusion “cloning” is a recombination system. We used it by following the In-Fusion® HD Cloning Kit User Manual (Clontech® Laboratories, Inc. No. 011614).

2.2.4 Purification of DNA fragment by gel

These experiments were carried out by using THERMO Scientific Gel extraction Kit (K0503) according to the kit’s instructions.

2.2.5 Ligation system

Ligation is performed by using T4 DNA ligase (Biolabs, No.M0202S) as follows:

T4 DNA ligase reaction buffer: 1×final

T4 DNA ligase: 0.5 μl

Vector: 0.1-1μM

Restriction fragment: 0.1-1μM

Deionized water to a total volume of 10 μl. Incubate the reaction mix at 16°C overnight.

2.2.6 Bacteria methods:

2.2.6.1 Thermo-competent cells preparation

Inoculate a single colony of *E.coli* to 50ml LB liquid medium, shaking overnight at 37°C. Next morning, dilute the culture to a start OD₆₀₀ 0.1 in a new flask (1L) containing 250ml LB medium. Cultivate under vigorous shaking at room temperature until OD₆₀₀ 0.6-0.8. Place flask on ice for 10 min. Centrifuge the culture at 3000rpm for 10 min at 4°C. Re-suspend cell pellets in 80 ml ice-cold TB buffer and incubate cells on ice for 10 min. Centrifuge at 3000 rpm for 10 min at 4°C, resuspend cells gently in 20 ML TB buffer and add DMSO to a final concentration of 7%. Incubate cells on ice for 10 min. Take 100 μl cells into microtubes and freeze them immediately in liquid nitrogen. Store at -80°C.

2.2.6.2 Transformation of thermo-competent bacteria

For one transformation, take 100 µl competent cells from -80°C and put on ice for 10 minutes. Add 2 µl plasmid DNA and leave on ice for another 20 minutes. Put the tube at 42°C for 60s-90s for the heat shock and put back on ice for another 5 minutes. Add 1ml LB medium and shake for 1 hour at 37°C. Centrifuge the culture, resuspend cells in 200 µl sterile water, spray on the selective agar and incubate at 37°C overnight.

2.2.6.3 Plasmid extraction

Inoculate one single *E.coli* colony to 5ml LB liquid medium + the appropriate antibiotic (ex : Ampicilin or Kanamycin), and cultivate with agitation overnight at 37°C. Collect cells from 3ml of the culture by centrifugation and remove the supernatant. Extract the plasmid by using THERMO plasmid miniprep kit (QIAGEN, No. k0692). After the extraction, the plasmid DNA concentration can be measured by the Nanodrop.

2.2.7 Yeasts Procedures:

2.2.7.1 Yeast Transformation by lithium acetate

Inoculate 2-5 ml of liquid YPD and incubate with shaking overnight at 30°C. Dilute the overnight liquid culture by 20 times in 50 ml falcon tube (1 ml overnight culture + 19 ml new YPD) and then incubate the new culture at 30°C on the shaker at 200 rpm for 4 hours. Harvest the culture in 1.5 ml eppendorf tube 2 times at 5000 rpm for 2 min (3 ml culture total for one transformation). Discard the supernatant and add 150 µl 100 mM LiAc. Mix the sample gently and leave it at room temperature 10 min. After centrifuging the mixture at 5000 min for 2 min, discard the supernatant and add the transformation mix. The transformation mix consists of:

- 90 µl PEG (50% w/v)
- 15 µl LiAc (1M)
- 5 µl ss-DNA (2.0 mg/ml)
- 1.5 µl plasmid

- 18 µl water

Mix with the pipet every time adding anew reagent. Incubate the mixture at 30°C for 45 min with agitation, then heat shock at 42°C for 15 min. After cooling it on ice for 5 min, centrifuge at 5000 rpm for 5 min. Discard the supernatant and resuspend cells in sterile water (150-200 µl). Plate all the cells on the selective plate (either SD agar with the appropriate autotrophy, or YPD plate containing G418).

2.2.7.2 Homologous Recombination

Homologous recombination (also known as general recombination) is a type of genetic recombination. Nucleotides can be exchanged between two similar or identical strands of DNA by homologous recombination. There are two different types of homologous recombination. One is involved in meiosis, which produces new combinations of DNA sequences during chromosomal crossover and another is involved in DNA repair during mitosis. Homologous recombination can be used as a technique in molecular biology, and is very useful for introducing genetic changes into genomic DNA of yeasts and other target organisms. Yeast cells can carry out homologous recombination that is an extremely efficient process. The constructs containing the gene of interest together with about 50 bp sequences at one end or both ends; which are homologous to the target site in the target vector or chromosome. The gene of interest can replace the sequence between these short homologs regions in the target vector or chromosome by recombination. Efficiency of this process could be greatly increased with gap-repair mechanism when the target is a vector, which is linearized between these two homologous regions. An advantage of recombination cloning is that the insert does not need to match the restriction enzyme sites on the vector.

The protocol for integrating transformations is similar to the yeast transformation. Substitute the plasmid by the target DNA in the protocol of yeast transformation above.

2.2.7.3 Extraction genome DNA

All genomic DNA have been isolated using the MasterPure™ Yeast DNA Purification Kit (Epicentre, No. MPY2-70921). Pellet the yeast cells from a saturated 1.5 ML culture by centrifugation in a microcentrifuge at 10000 rpm for 2-5 min. Remove all growth medium and add 300µl of Yeast Cell Lysis Solution to each microcentrifuge tube suspend the cells by

vortex mixing or pipetting the cells repeatedly using a 1 ml capacity pipet tip. Incubate the suspended cells at 65°C for 15 minutes. Place the samples on ice for 5 minutes. Add 150 µl of MPC Protein Precipitation Reagent and vortex mix for 10 seconds. Pellet cellular debris by centrifugation in a microcentrifuge for 10 minutes at 10,000 rpm. Transfer the supernatant to a clean microcentrifuge tube and add 500 µl of isopropanol. Mix thoroughly by inversion. Pellet the DNA by centrifugation in a microcentrifuge for 10 minutes at 10 000 rpm. Remove the supernatant by pipeting and discard. Wash the pellet containing the DNA with 0.5 ml of 70% ethanol. Carefully remove the ethanol by pipetting and discard. Briefly centrifuge the DNA pellet and remove any remaining ethanol. Suspend the DNA in 35 µl of TE Buffer. Store the DNA at 4°C.

2.2.7.4 Yeast two-hybrid system

The principle of the Two-hybrid system is described in the Figure 15. It is based on the functional reconstitution of an intact transcription factor that activates reporter gene expression (Figure 15). Reporter gene expression can efficiently be selected for in yeast. As indicated by the name, the yeast two-hybrid system utilizes two hybrid proteins. The bait protein (X; Figure 15) is fused to the DNA binding domain (BD; Figure 15) of a transcription factor. The prey protein (Y; Figure 15) is fused to the transcription activation domain (AD; Figure. 15) of a transcription factor. In our system, we use the yeast transcription factor Gal4. When two hybrid proteins are co-expressed in yeast and when X and Y can physically interact with each other, and a functional transcription factor is reconstituted. This reconstituted transcription factor activates the expression of a set of reporter genes (in our case *ADE2*, *HIS3* and LacZ encoding the enzyme beta-galactosidase).

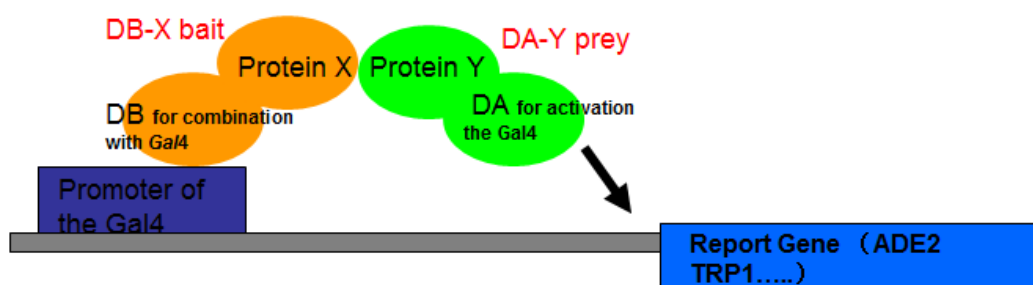


Figure 15: The Two hybrid system

2.2.7.5 The procedure

Take the strain PJ694A from stock – 80°C on the plate YPD and incubate at 30°C until the strain grows (2-3 days). Inoculate colons from the YPD plate into YPD liquid medium shaking at 200 rpm at 30°C for overnight. The second morning, do the transform plasmids PGAD424, PGAD424-TYS1 and PGAD424-CNA1 into PJ694A individually and grow on the Leu- SD solid plate at 30°C for growing. Until the transformers grow well, pick the clones into Leu- SD liquid medium shaking at 200 rpm at 30°C for overnight. And the second morning transform plasmids POBD80, POBD80-KNR4, POBD80-KNR4^{S200A203A} and POBD80-KNR4^{S200D203D} into 3 transformers separately and grow on Leu-Trp- SD liquid plates.

Table 4. Table for the two-hybrid system

Plasmid	GalBD	GalBD- <i>knr4</i>	GalBD - <i>knr4</i> ^{S200A203A}	GalBD- <i>knr4</i> ^{S200D203D}
GalAD	1.PGAD424 & POBD80	2. PGAD424& POBD80- <i>knr4</i>	3.PGAD424& POBD80- <i>knr4</i> ^{S200A203A}	4.PGAD424& POBD80- <i>knr4</i> ^{S200D203D}
GalAD-TYS1	5.PGAD424-TYS1& POBD80	6.PGAD424-TYS1& POBD80- <i>knr4</i>	7.PGAD424-TYS1& POBD80- <i>knr4</i> ^{S200A203A}	8.PGAD424-TYS1 & POBD80- <i>knr4</i> ^{S200D203D}
GalAD-CNA1	9.PGAD424-CNA1 & POBD80	10. PGAD424-CNA1 & POBD80- <i>knr4</i>	11. PGAD424-CNA1 & POBD80- <i>knr4</i> ^{S200A203A}	12.PGAD424-CNA1& POBD80- <i>knr4</i> ^{S200D203D}

Inoculate all these 12 transformants separately on a new Leu-Trp- SD plate at 30°C for 2 days. And then copy the strains on His- and Ade- SD plate at 30°C.

Inoculate all these 12 transformants separately into liquid Leu-Trp- medium at 30°C overnight. Second morning concentrate 5ml culture at 5000 rpm for 2 min and keep the cell pellet for measuring β -galactosidase activity. β -galactosidase activity is measured from protein extracts f using the chromogenic substrate ONPG.

2.2.8 Protein- Western-blot

2.2.8.1 SDS – Page Protein

Add 20 μ l 6 X Loading buffer with the 100 μ l protein sample into eppendorf tube, boil the mixture at 100°C for 5 min and then put on the ice for 5 min. Load 10-50 μ l protein sample in the well of the SDS-PAGE gel (Mini-PROTEAN TGX Gel, BIO-RAD No. 456-1034) and 5 μ l PageRuler Prestained Protein Ladder (Fermentas, No.00012835) as the marker was added

in the well of. Migration of the protein was performed in the migration buffer 1 × dilution of the NEST GEL RUNNING BUFFER 20 × (AMRESCO, No.2487B033) at constant voltage of 100V. After migration, the polyacrylamide gel passed to the transfer system for immunoblotting or stain with the dye PageBlure Protein Staining Solution (Fermentas, No.R0571)

2.2.8.2 Transfer to Nitrocellulose membrane

This experiment was performed by the Trans-Blot Electrophoretic Transfer Cell system(RIO-RAD). Cut the gel to fit the membrane and carefully place the gel on top of the transfer membrane (Trans-blot Turbo Transfer Pack Mini format 0.2 μm, BIO-RAD No.170-4158) and put the filter paper on top of the gel carefully removing air bubbles from between the gel and filter paper, Turn on the power supply and transfer for 10 min.

2.2.8.3 Immune blotting

Primary anti-bodies: One antibody is 4A2 which epitope is unknown (Biol. Pharm. Bull. 27(5), 691-693, 2004) and the other is killer toxin neutralising antibody, nmAb-KT, whose epitome is 41GSTDGK46 (J. Biochemistry 139, 399-406, 2006).

Second anti-bodies: Anti-mouse IgG (whole molecule) Alkaline Phosphatase in Goat, A3526-.25ml. Dilute in the Blocking buffer or 5% (W/V) milk.

Blocking buffer: SuperBlock Blocking Buffer in PBS No.37515 and SuperBlock Blocking Buffer in TBS No.37535

Dilution buffer: 1/5 × Blocking buffer or 5% without-fat milk (W/V solute in PBS or TBS).

Washing buffer: 1 × PBS or 1 × TBS.

Remove the blot from the transfer apparatus. Put the gel into the dye and put the membrane into the blocking buffer for 60 minutes at room temperature with shaking. Remove the blocking buffer and add the appropriate primary antibody dilution. Incubate membrane for 1 hour with shaking at room temperature or incubate it at 4°C overnight. Wash membrane in Wash Buffer by agitation for 5 minutes and repeat 5 times. Incubate the secondary anti-bodies for 1 hour. And wash 5 times again. Put membranes in one clean plate and add 1-2 ml

BCIP/NBT-Purple Liquid Substrate System for membrane (SIGMA, No. B3679-100ml) to the blot.

2.3 Phenotype test on the plate

2.3.1 The preparation of the plate containing the different toxin for drugs

The solid medium is prepared as introduced. Once the temperature of the medium is below the 60°C add the different concentration of the calcofluor white, congo red, caspofungi and K9.

Calcofluor white

Prepare 10 ml 5 mg/ml the solution of the calcofluor white. Weight 50 mg powder of the calcofluor white (Sigama, No. F - 3543) and put into 14 ml sterilized falcon tube. Add 4 ml water in it and vortex 10 seconds. Now the mixture is muddy. Add drops of 10 M NaOH and vortex until the solution became clear. And then add the water until to 10 ml. And then filter the solution by 0.2 µm film into the new falcon tube wrapped by the silver paper avoiding the light. The solution can be saved at 4°C for 5 days.

Add the calcofluor white into the solid medium for final concentration 0.1mg/ml, 0.05mg/ml and 0.01mg/ml.

The Congo red

Prepare 5 ml 10 mg/ml the solution of the calcofluor white. Weight 50 mg powder of the Congo red (Sigama; No. C - 6767) and put into 14 ml sterilized falcon tube. Add 5 ml water in it and vortex. And then filter the solution by 0.2 µm film into the new falcon tube. The solution can be saved at 4°C for 1 week.

Add the Congo red solution into the solid medium for final concentration 0.25mg/ml, and 0.1mg/ml.

The caspofungi

Add the caspofungi solution into the solid medium for final concentration 100 µg/ml, and 200 µg/ml.

2.3.2 Procedure for the phenotype test

Phenotypic screening is a type of screening used in biological research and drug discovery to identify substances such as small molecules, peptides, or RNAi that alter the phenotype of a cell or an organism in a desired manner. Here we do the phenotypic yeast growth analysis of the wildtype and the mutants for calcofluor white, congo red, K9 killer toxin caspofugin toxicity testing.

Inoculate the interesting strains in the liquid medium and incubate at 30°C 200rpm overnight. The second morning measure the OD₆₀₀ and adjust the cell density at OD₆₀₀=8 by concentration and dilution by sterilized water. And dilute the culture 10 times gradiately (1, 1/10, 1/100, 1/1000). Drop 2.5µl culture of different dilutions on the different test plates. These plate are incubated at 30°C for 2 days. And the test sensibility of temperature is performed on the normal YPD plate at 30°C.

And for the complementation phenotype test, the strains interested are *knr4* mutants which contains different plasmid (different genotype of the *KNR4*) individually.

2.3.3 Halo test for the K9

2.3.3.1 The preparation of the K9

Killer K9 toxin, also referred to as HM-1, is secreted in the culture supernatant of the strain *Hansenula mrakii* IFO0895 obtained from ATCC. This strain is cultured for 72 hours at 19°C in liquid YPD adjusted to pH4.3 by the addition of 5 mM Na citrate. Cells are pelleted by centrifugation, and the supernatant is filtered on 0.22 µm pores filter. Further concentration of the toxin is performed using Amicon ultra-15 centrifugal filter device at 4°C, for 40-60 minute, 5 times. At last about 60 mL culture supernatant is concentrated in 1ml. The final toxin concentration is estimated by Bradford method. The concentrated K9 killer toxin has been checked by SDS-PAGE, resulting in one clear band of the killer toxin of 9.5 Da size.

2.3.3.2 The determination of the K9 Minimal Inhibitory Concentration (MIC)

The determination of Minimal Inhibitory Concentration (MIC) of K9 killer toxin against control *Saccharomyces cerevisiae* strain BY4741 was determined according to the protocol provided by the Clinical Laboratory Standard Institute (CLSI)(CLSI 2008). Briefly, a yeast

solution of $OD_{600}=0.150$ was prepared from fresh agar plate. This cell suspension was then diluted to obtain a concentration in cells 5×10^2 - 2.5×10^3 cells/ml. Different concentrations of K9 killer toxin (from $2.5 \mu\text{g/mL}$ to $80 \mu\text{g/mL}$) were placed in tubes, containing 0.9 mL of the yeast suspension. For the control, 0.1 mL of sterile water was used instead of K9. Cultures were incubated at 30°C for 24h, before reading OD_{600} in order to determine the concentration of the K9 killer toxin needed to inhibit growth. This test was repeated 3 independent times with the same result. The MIC of K9 killer toxin was determined as 20 mg/L.

2.3.3.3 Halo test for the K9 on the solid plate

Killer toxin halo tests are performed on YPD plate at 30°C overnight. First we inoculate clones of different strains into YPD or SC liquid medium at 30°C overnight. Cell density is adjusted to $OD_{600}=4$ by centrifugation and/or dilution in sterile YPD medium. 200 μl yeast solution is sprayed on the agar plate (Petri dish containing solid YPD medium). After 1 h drying by incubation at 30°C , K9 killer toxin solution at a concentration of 0.8 mg/mL is spotted on the plate, as independent dots of different volumes (10 and 20 μl). Alternatively, larger volumes (30 μl) can be used. Petri dishes are incubated at 30°C overnight.

2.4 Methods and systems for the signalling pathway

2.4.1 Method for measuring the CWI pathway

For measuring the CWI signalling, here we introduce the most simpler one for measuring sustained signalling through the CWI pathway is to employ *lacZ* reporter gene which is driven by Rlm1-responsive promoters (Jung et al. 2002). The plasmid YIL117C-LacZ that contains duplicated binding site from YLC117C fused to the minimal CYC1 promoter is transformed into the different strains on URA- SD plate at 30°C . And then inoculate the clone into liquid URA- SD medium at the 23°C . Dilute the culture to 0.1 of OD_{600} in YEPD. The culture was grown to mid-log phase at 23°C prior to induction of calcofluor white for the 5h. And then β -galactosidase activity was measured from protein extracts using the chromogenic substrate ONPG.

2.4.2 Method for measuring the calcinerin signalling pathway

pSG2, pAL3 and pAL5 plasmids, constructed in the laboratory by Arnaud Lagorce and Samuel Gelis, were used to test the calcineurin signalling pathway activation. These plasmid

take advantage of the fact that the transcription factor Crz1, downstream element of the calcineurin pathway, binds specific DNA sequences in target gene promoters, named CDREs (calcineurin-dependent response elements) or WCE (wall consensus element). The core consensus site for Crz1 binding was shown to be GNGGC(G/T)CA (Stathopoulos & Cyert 1997; Yoshimoto et al. 2002). pSG2 was constructed from the plasmid of pLG669 (vector Amp, *URA3*; fusion with pCYC1- LacZ), in which 6WCE-elements were added by PCR. We used pSG2 to transform the strains BY4741, *knr4* mutant, *knr4S200AS203A* and *knr4^{S200DS203D}*.

The pAL3 and pAL5 plasmids both contain lacZ gene under the dependence of *FKS2* promoter. *FKS2* gene encodes an alternative subunit of the yeast β -(1,3)-glucan synthase. The promoter of this gene responds to various stresses and is controlled by several signal transduction pathways, including Calcineurin pathway. In the pAL3, the WCE element was removed from the *FKS2* promoter, while 6 WCEs copies have been integrated by PCR in pAL5 *FKS2* promoter. The plasmid pAL3 is used here as a negative control for Calcineurin induced transcriptional activity.

All these plasmids were transformed into the 7 following strains: *knr4^{S200AS203A}*, *knr4^{S200DS203D}*, *knr4 Δ N*, *knr4 Δ N+C*, *knr4 Δ C*, BY4741 and *knr4 Δ* , which were then grown on URA- SD plate at 30°C. These clones were then inoculated into liquid URA- SD medium, with shaking at the 30°C overnight. The second morning, we measured the OD of the culture and dilute the culture to 0.1 of OD₆₀₀ in YPD. The culture was grown to mid-log phase at 30°C prior to induction by Ca²⁺ (0.2M CaCl₂) for 30 min. And then β -galactosidase activity was measured from protein extracts using the chromogenic substrate ONPG.

2.4.3 Complementation test of the synthetic lethality

The BEM1, BCK1 and SLT2 has been known are synthetic lethal with the KNR4. So the Δ *bem1 knr4*, Δ *bck1 knr4* and Δ *slt2 knr4* are saved with the plasmid expressing Knr4 (select marker *URA3*). Transform the different forms of Knr4 (phosphorylation site mutants select marker *LEU*) into these mutants on the LEU- and URA- SD plate. First pick the several single-clones of different transformations on the LEU- and URA- SD plate and then copy the plate on the 5-FOA plates for showing the complementation ability of the mutants and on the LEU- plate for the control.

2.5 Dosage biochimiques

2.5.1 Chitin measurement

Preparation of cells: First- do preculture of strain (in 5 ml/YPD- put it on mixing (200 rpm t 30°C for 1 day)- **triplicate**. After 1 day, check OD₆₀₀ nm and dilute it to 0.1. Cultivate cells in 200 mL of medium under 200 rpm until OD₆₀₀=1 (check OD₆₀₀ nm every 2 hours). Divide it in 4 Erlenmeyer flask. Harvest cells by centrifugation (10 min at 4000 rpm, 21 degree). Wash cells 2 times with 5 ml sterilized cold water until pellets are clear (centrifuge after washing- 4000rpm/5min/4 degree). Store the pellets at -20°C, or continue.

Isolation of cell walls: Resuspend the pellet in 500 µL of 10 mM TrisCl + 1mM EDTA pH 7.5. Transfer in tubes containing beads (MPBio, 6960). Break with a FastPrep system by several cycles of 20 sec at max speed (6.5 m/s) and 1 min on ice during each cycle. Check the breakage at microscope by blue methylene test coloration. Transfer the cell suspension in a falcon tube of 15 mL, without taking the beads. Wash 4 times with 1 ml of cold water and collect each time the supernatant. Centrifuge falcon tubes for 10 minutes at 4000 rpm in 4°C. Remove the supernatant. At the end, wash cell walls from all Falcon tubes with 1 ml of cold water, divide it in 2 parts, and centrifuge again (4000rpm/10 min/4 degree). Remove supernatant and Lyophilisate the cell walls with the freeze-dry system (put before in liquid N₂). Wait for minimum 5 days to dry cell walls.

Then, start with **enzymatic hydrolysis**: Weight around 10 mg of purified cell walls (do duplication or triplication) in eppendorf tube. In that tube add 200 µl 50 Mm potassium acetate pH=5. Vortex and put it in water bath 60 °C for 5 min. Vortex again and add 25 µL of chitinase (4 U/ml) + 40 µL endo-beta-1,3-glucanase (50 U/ mL) + 20 µl exo-beta-1,3-glucanase (100 U/mL). Put it in mixing machine, in room of 37°C for 24 hours. After one day take 50 µL of aliquot to measure first step (dilute it 800x, first 40, than 20 times, put it in vials and store in -20 °C, or give it to analysis)

At rest of sample (235 µl) add 25µl beta-glucosidase and 40 µL endo-1, 6- glucanase. Put it also in mixing machine at 37°C for 24 hours. After take also aliquot for measure step 2 of enzymatic hydrolysis, and dilute it also 800 times. Rest of samples store at -20°C. At the end do the Reissig's method, which is method to calculate amount of chitin:

Reissig's method

Reagent:

Standard of N-acetyl-D-glucosamine at 10 mM: Dissolve 44.2 mg in 20 ml of water (check pH~7).

Solution of Tetraborate Potassium 0.8 M pH 9,0: Dissolve 6.11 g in 25 ml of water. Adjust pH with KOH 3M.

Reissig's reagent 10X : Dissolve 10 g of 4-diméthylaminobenzaydedyde (Fluka) in 12.5 ml of HCl 10 N and 87.5 ml of glacial acetic acid (Store in fridge; sensitive to light and keep during 2 months).

Note: For the dosage the reagent is diluted 10 times in acetic acid.

Transfer 125 µl of enzymatic mixture or standard in tubes and add 25 µl of potassium tetraborate 0.8 M pH 9.0. Heat at 100 °C for 8 min the tubes. Make them cold at room temperature. Add in tubes 750 µl of Reissig Reagent 1X (from the 10X solution). Incubate immediately tubes at 37 °C for 40 min. Read the DO at 550 nm. Make a standard curve of N-acetyl-D-glucosamine: 0 à 0.5 mM from the 10 mM stock solution.

Standard curve:

100 µl de standard solution at 10 mM in 1 ml of water → 1 mM

200 µl de standard solution at 1 mM in 200 µl of water → 0.5 mM

200 µl de standard solution at 0.5 mM in 200 µl of water → 0.25 mM

100 µl de standard solution at 1 mM in 1 ml of water → 0.1 mM

200 µl de standard solution at 0.1 mM in 200 µl of water → 0.05 mM

200 µl de standard solution at 0.05 mM in 200 µl of water → 0.025 mM

100 µl de standard solution at 0.1 mM in 1 ml of water → 0.010 mM

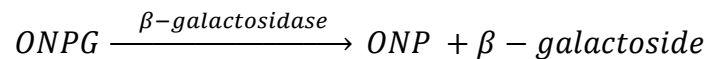
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At the end we can get Chitin (µg/mg)= concentration of GlnAc; Reissig's method

2.5.2 β -galactosidase activity

Extraction the β -galactosidase: Concentrate the 8 ml culture of each strain and keep the pellet. Resuspend the cell pellet in 250 μ l Z buffer (with 1 mM DTT). Put the cells in a glass tube with a thick wall and add 250 mg of glass beads (diameter 0.5 mm). Vortex during 30 seconds and cool the tube during 1 min in ice and repeat this breaking step 5 times. Centrifuge at 2000 rpm for 10 min at 4°C. Use the supernatant to measure β -galactosidase activity.

Assay: At the time t_0 , one volume of extract (20-50 μ l) is mixed with 400 μ l 4 mg/ml ONPG that is preheated at 37°C. A yellow colour appears if the reaction occurs. When the mix is straw coloured; stop the reaction at the time t , by adding 1 ml of Na₂CO₃ 1M and keep the tube at 4°C. The reaction should not be stopped too prevent a saturation of the colorimeter. A control is realised in parallel by adding water instead of the extract. Put 200 μ l of each sample on an ELISA plate and measure the coloration at 420 nm with the MR7000 Dynatech reader. Detection limits are between A_{420} 0.01-0.3. And then calculate the Specific Activity.



$\epsilon(\text{ONP}) = 4500 \text{ L} \cdot \text{mol}^{-1} \cdot \text{cm}^{-1}$ at 420 nm;

$A_{420} = 1$ corresponds to 222 nmol ONP produce in 1 ml of reaction mix

$$AS = A_{420} \times 222 \times \frac{1000}{\text{extract volume}(\mu\text{l})} \times \frac{1}{[t_x - t_0](\text{min})} \times \frac{1}{\text{mg proteins/ml}}$$

2.5.3 Bradford method for the protein

10mg/ ml commercial BSA solution. Dilute the BSA (50 μ g/ml, 35 μ g/ml, 25 μ g/ml, 12.5 μ g/ml, 6.25 μ g/ml) and dilute the protein extraction (1/100, 1/200, 1/400). Add 200 μ l samples into each well of the Elisa plate and then add the 50 μ l brandford reaction. Mix with pipette and put it in the black for 10 minutes. Read the OD550 and calculate the protein concentration.

2.6 Cytometry experiments for the K9 Treatment cells

BY4741 and $\Delta knr4$ grow to OD600 0.6 and treat by $\frac{1}{2}$ MIC and 4MIC K9 treated cells of BY4741 and $\Delta knr4$ were washed and diluted to OD600 0.1 by PBS. And as control, BY and $\Delta knr4$ without K9 treatment are added YPD medium. 10^5 cells were analysed by the Attune™ Acoustic Flow Cytometer (Life Technologies). The SSC/FSC dot plots were created by

the Attune™ software (Life Technologies). The mean FSC value of each sample was recorded to compare the cell size.

2.7 Microscopy

2.7.1 Fluorescent Microscopy

We transformed pHM43 bearing a fusion protein Knr4 with GFP into the deletion mutants selected, Cells were grown in SD medium supplemented with auxotrophic requirements, and observed either directly or after exposure to 7.5 µg/ml synthetic α -factor (WHWLQLKPGQPMY; Sigma). For the assay of shmoo formation exponentially growing cells in YPD (OD₆₀₀ = 0.5) were exposed to 7.5 µg/ml α -factor (Sigma) and the progression of shmoo formation objective, HCX PL APO CS. 1.40 oil) with excitation filters 421-450 nm and emission filter 466-500 nm.

The plasmid expressing Knr4^{S200D203D} with GFP (pUG35-Knr4^{S200D203D}) was transformed in the Knr4 null mutant and By4741 and observed under the fluorescence microscopy. PHM43 (pUG35-Knr4-GFP) was used as control. The cells were observed under a Leica DM4000B microscope with excitation filters 421-450 nm and emission filter 466-500 nm.

2.7.2 AFM

Culture of yeast cells was done as described above. After collecting treated and non-treated cells by centrifugation, and washed two times with acetate buffer (18 mM Na Acetate, 1 mM CaCl₂, 1 mM MnCl₂, pH=5.2), cells were resuspended in the same buffer at OD₆₀₀=1.0, and immobilized in polydimethylsiloxane (PDMS) stamps prepared as described previously(Dague et al. 2011). Briefly, freshly oxygen activated microstructured PDMS stamps were covered by a total of 100 µL of the cell suspension and allowed to stand for 15 minutes at room temperature. Convective/capillary assembly then deposited the cells into the structures of the stamp. AFM Images were recorded in Quantitative Imaging™(Chopin et al. 2013) mode on a Nanowizard 3 AFM (JPK Instruments, Berlin, Germany), with MLCT AUWH cantilevers (nominal spring constant of 0.01 N/m, Bruker, USA). The cantilevers spring constants were determined by the thermal noise method (Hutter & Bechhoefer 1993) prior to each experiments. The applied force used in the AFM Imaging mode was kept at 1.0 nN. For nanomechanical properties measurements, force maps of 32 ×32 force curves were recorded on areas of 1 µm × 1 µm on top of the cells. The force-distance curves recorded

were transformed into force-indentation curves by subtracting the cantilever deflection on a solid surface. The indentation curves were then fitted to the Hertz model, which links the force (F) as a function of the elastic modulus (E) and the square of the indentation (δ) for a conical indenter.

$$F = \frac{2E \tan \alpha}{\pi(1-\nu^2)} \delta^2 \quad [1]$$

In equation [1], α is the tip opening angle (17.5°) and ν the Poisson ratio assumed to be 0.5.

Chapter 3: Results and Discussion

3.1 Localization of Knr4 in the morphogenesis genes deletion mutants

In the control *S. cerevisiae* strain BY4741, a C-terminal fusion of Knr4 with the Green Fluorescent Protein GFP (Chalfie *et al.*, 1994) localizes at the presumptive bud site in G1, then at the tip of small buds and finally at the mother-daughter neck during cytokinesis (Martin *et al.*, 1999). Knr4 N-terminal domain is required for the correct cellular localization of the protein, since this localization is lost for the GFP fusion of Knr4 (80–505) lacking this domain. This result suggests that Knr4 interacts through this N-term domain with at least one protein involved in its localization. Similarly, the N-terminal domain is required for the correct localization of Knr4 at the shmoo tip during sexual differentiation. The sites of Knr4 localization at the bud tip and the shmoo tip are also the place of polarized growth. The initial polarization of Cdc24p and Cdc42p during shmoo-site and bud-site selection depends strongly on Bem1p. Bem1p colocalizes with Cdc24p and Cdc42p to growth sites, and its transcription in G1 coincides with these early polarization events (for a review Pruyne & Bretscher, 2000). We established earlier that Knr4 can interact physically with proteins directly involved in polarized growth including Bem1 and Act1 (Fadi Basmaji *et al.* 2006). In this study, we wished to go further and have a global view of the Knr4 proteins partners involved in yeast cell morphogenesis and polarized growth.

In order to find out these unknown partners and to investigate the cellular pathways required for localization of Knr4 protein, we decided to use a series of deletion mutants interrupted in genes related to morphogenesis and establishment of cellular polarity. We selected candidate genes from the *Saccharomyces cerevisiae* genome database (SGD, Stanford), using the keywords “Morphogenesis” and “Cell Polarity”. This screen yielded 29 different genes, to which we added *BNII* and *BNRI* encoding the two *S. cerevisiae* formins (required for the formation of the actin cables) and *SPA2* and *BUD6*, encoding members of the polarisome. From these 33 different genes, we eliminated the essential ones (since we could not use their deletion mutants), as well as the ones encoding proteins known to be localized elsewhere in the cell (*i.e.*: away from the sites of polarized growth and the Knr4-GFP fusion protein). 27 genes were kept for our analysis. Out of these 27, 7 were unfortunately absent from the Yeast Knock Out (YKO) collection available in the lab. The 20 mutants deleted in the remaining

candidate genes were then transformed with a plasmid bearing a Knr4-GFP fusion protein, and we verified whether the localization of Knr4 was conserved, both among the cell cycle (vegetative growth) and during sexual differentiation. We used plasmids that express Knr4 fused to GFP (pHM43) and control plasmid expressing the GFP without Knr4 (pUG35). After transformation, the different mutants were observed by fluorescence microscopy.

After a thorough analysis of the results we got first, we decided to also observe Knr4-GFP localization in other mutants correlated to the first mutants, like *cdc55*, *pph21*, *pph22* (related to *tpd3*). As we know Knr4 is involved in the CWI pathway and calcinerin pathway, we also checked the localization of Knr4 in the mutants *cna1Δ* and *slt2Δ*. Finally, we tested the Knr4 localization in the shmoo tips and bud tips of a total of 25 mutants.

3.1.1 Localization of Knr4-GFP expressed from plasmid in the 20 mutants

We studied the localization of Knr4-GFP fusion protein expressed from centromeric plasmid pHM43 in the 20 selected mutants, which are described in Table 5.

Through the observation by fluorescence microscopy we verified whether the localization of Knr4 was conserved during vegetative growth among the cell cycle, as well as during sexual differentiation.

Table 5: Details and function of the 20 genes selected for this study

N°	GENE	SYSTEMATIC NAME	Function
1	<i>ACE2</i>	YLR131C	Transcription factor required for septum destruction after cytokinesis; phosphorylation by Cbk1p blocks nuclear exit of Ace2p during the M-to-G1 transition, causing its specific localization to daughter cell nuclei, and also increases Ace2p activity; phosphorylation by Cdc28p and Pho85p prevents nuclear import during cell cycle phases other than cytokinesis; part of the RAM network that regulates cellular polarity and morphogenesis
2	<i>BEM1</i>	YBR200W	Protein containing SH3-domains, involved in establishing cell polarity and morphogenesis; functions as a scaffold protein for complexes that include Cdc24p, Ste5p, Ste20p, and Rsr1p.
3	<i>BEM2</i>	YER155C	Rho GTPase activating protein (RhoGAP) involved in the control of cytoskeleton organization and cellular morphogenesis; required for bud emergence
4	<i>BNI1</i>	YNL271C	Formin, nucleates the formation of linear actin filaments, involved in cell processes such as budding and mitotic spindle orientation which require the formation of polarized actin cables, functionally redundant with BNR1

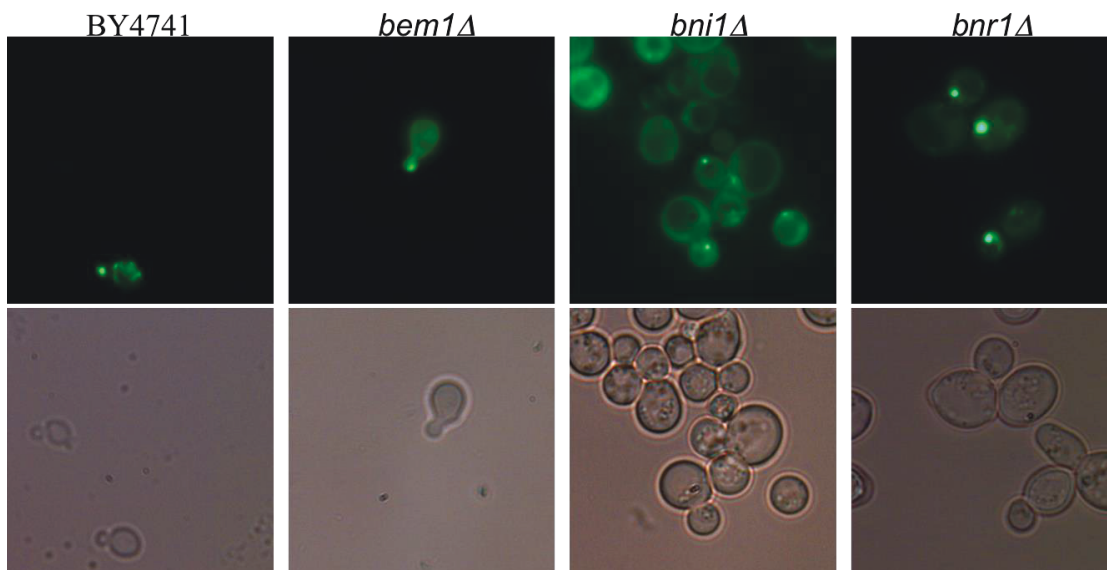
5	<i>BNR1</i>	YIL159W	Formin, nucleates the formation of linear actin filaments, involved in cell processes such as budding and mitotic spindle orientation which require the formation of polarized actin cables, functionally redundant with BNI1
6	<i>BUD6</i>	YLR319C	Actin- and formin-interacting protein; stimulates actin cable nucleation by recruiting actin monomers to Bni1p; involved in polarized cell growth; isolated as bipolar budding mutant; potential Cdc28p substrate
7	<i>ELM1</i>	YKL048C	Serine/threonine protein kinase that regulates cellular morphogenesis, septin behavior, and cytokinesis; required for the regulation of other kinases; forms part of the bud neck ring
8	<i>HBT1</i>	YDL223C	Substrate of the Hub1p ubiquitin-like protein that localizes to the shmoo tip (mating projection); mutants are defective for mating projection formation, thereby implicating Hbt1p in polarized cell morphogenesis
9	<i>HSL1</i>	YKL101W	Nim1p-related protein kinase that regulates the morphogenesis and septin checkpoints; associates with the assembled septin filament; required along with Hsl7p for bud neck recruitment, phosphorylation, and degradation of Swe1p
10	<i>PAMI</i>	YDR251W	Essential protein of unknown function; exhibits variable expression during colony morphogenesis; overexpression permits survival without protein phosphatase 2A, inhibits growth, and induces a filamentous phenotype
11	<i>PEA2</i>	YER149C	Coiled-coil polarisome protein required for polarized morphogenesis, cell fusion, and low affinity Ca ²⁺ influx; forms polarisome complex with Bni1p, Bud6p, and Spa2p; localizes to sites of polarized growth
12	<i>PCL1</i>	YNL289W	Cyclin, interacts with cyclin-dependent kinase Pho85p; member of the Pcl1,2-like subfamily, involved in the regulation of polarized growth and morphogenesis and progression through the cell cycle; localizes to sites of polarized cell growth
13	<i>PCL2</i>	YDL127W	Cyclin, interacts with cyclin-dependent kinase Pho85p; member of the Pcl1,2-like subfamily, involved in the regulation of polarized growth and morphogenesis and progression through the cell cycle; localizes to sites of polarized cell growth
14	<i>RRD1</i>	YIL153W	Peptidyl-prolyl cis/trans-isomerase; activator of the phosphotyrosyl phosphatase activity of PP2A; involved in G1 phase progression, microtubule dynamics, bud morphogenesis and DNA repair; required for rapid reduction of Sgs1p levels in response to rapamycin; subunit of the Tap42p- Sit4p-Rrd1p complex
15	<i>SPA2</i>	YLL021W	Protein involved in the transport of cell wall components from the Golgi to the cell surface; required for bud growth
16	<i>SBE2</i>	YDR351W	Protein involved in the transport of cell wall components from the Golgi to the cell surface; similar in structure and functionally redundant with Sbe2p; involved in bud growth
17	<i>SBE22</i>	YHR103W	Component of the polarisome, which functions in actin cytoskeletal organization during polarized growth; acts as a scaffold for Mkk1p and Mpk1p cell wall integrity signalling components; potential Cdc28p substrate
18	<i>TPD3</i>	YAL016W	Regulatory subunit A of the heterotrimeric protein phosphatase 2A (PP2A), which also contains regulatory subunit Cdc55p and either catalytic subunit Pph21p or Pph22p; required for cell morphogenesis and transcription by RNA polymerase III

19	<i>YCK1</i>	YHR135C	Palmitoylated plasma membrane-bound casein kinase I isoform; shares redundant functions with Yck2p in morphogenesis, proper septin assembly, endocytic trafficking; provides an essential function overlapping with that of Yck2p
20	<i>YCK2</i>	YNL154C	Palmitoylated plasma membrane-bound casein kinase I isoform; shares redundant functions with Yck1p in morphogenesis, proper septin assembly, endocytic trafficking; provides an essential function overlapping with that of Yck1p

3.1.1.1 During the vegetative growth, these 20 mutants may be divided into three groups according to their pattern of localization of Knr4-GFP:

- Localization at the bud tip in G1 like in the wild type (Figure 16)
- Abnormal localization of Knr4 (Figure 17)
- Abnormal cell shape (Figure 18)

In deletion mutants of 10 genes, *BEM1*, *BNI1*, *BNR1*, *BUD6*, *HBT1*, *PAMI*, *PEA2*, *SBE2*, *SBE22* and *YCK2*, Knr4-GFP concentrates in the bud tip in G1.



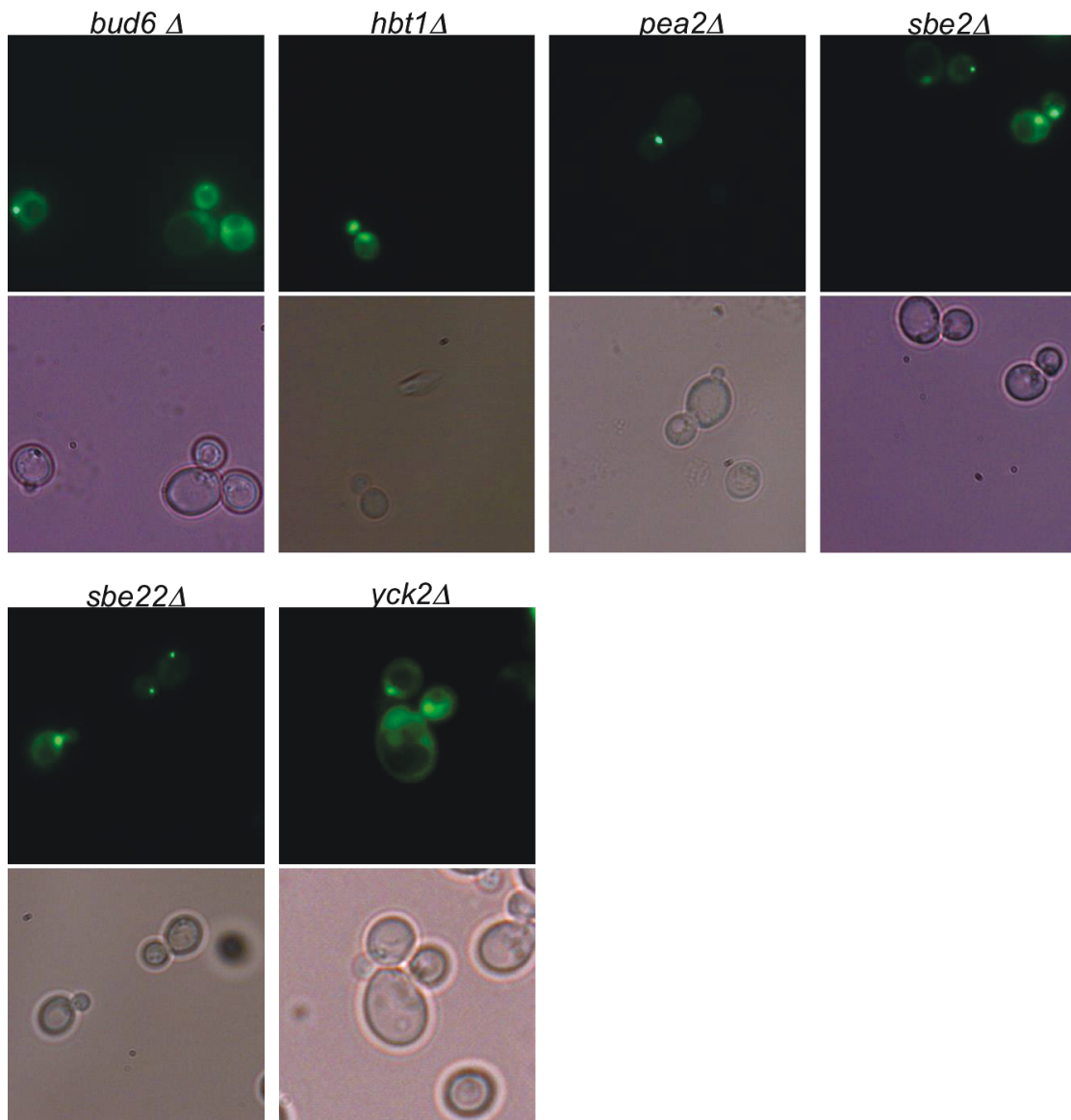


Figure 16: BY4741 and 9 of 20 mutants in which the localization of knr4 is normal

In deletion mutants of *YCK1* Knr4-GFP seems disperse within the cell, although there are some bright spots. And in the mutant of *BEM2*, *RRD1*, *PCL1*, *TPD3*, *PCL2*, *SPA2* and *YCK1*, the localization of Knr4 is abnormal. In *BEM2*, *PCL1*, *PCL2*, *SPA2*, *YCK1* and *PAM1* there is Knr4 protein concentration (*i.e.*: protein patches can be observed), but less concentrated than BY4741. In *TPD3* mutant, there are some small and bright points (like patches) in the cells (G1, budding cell).

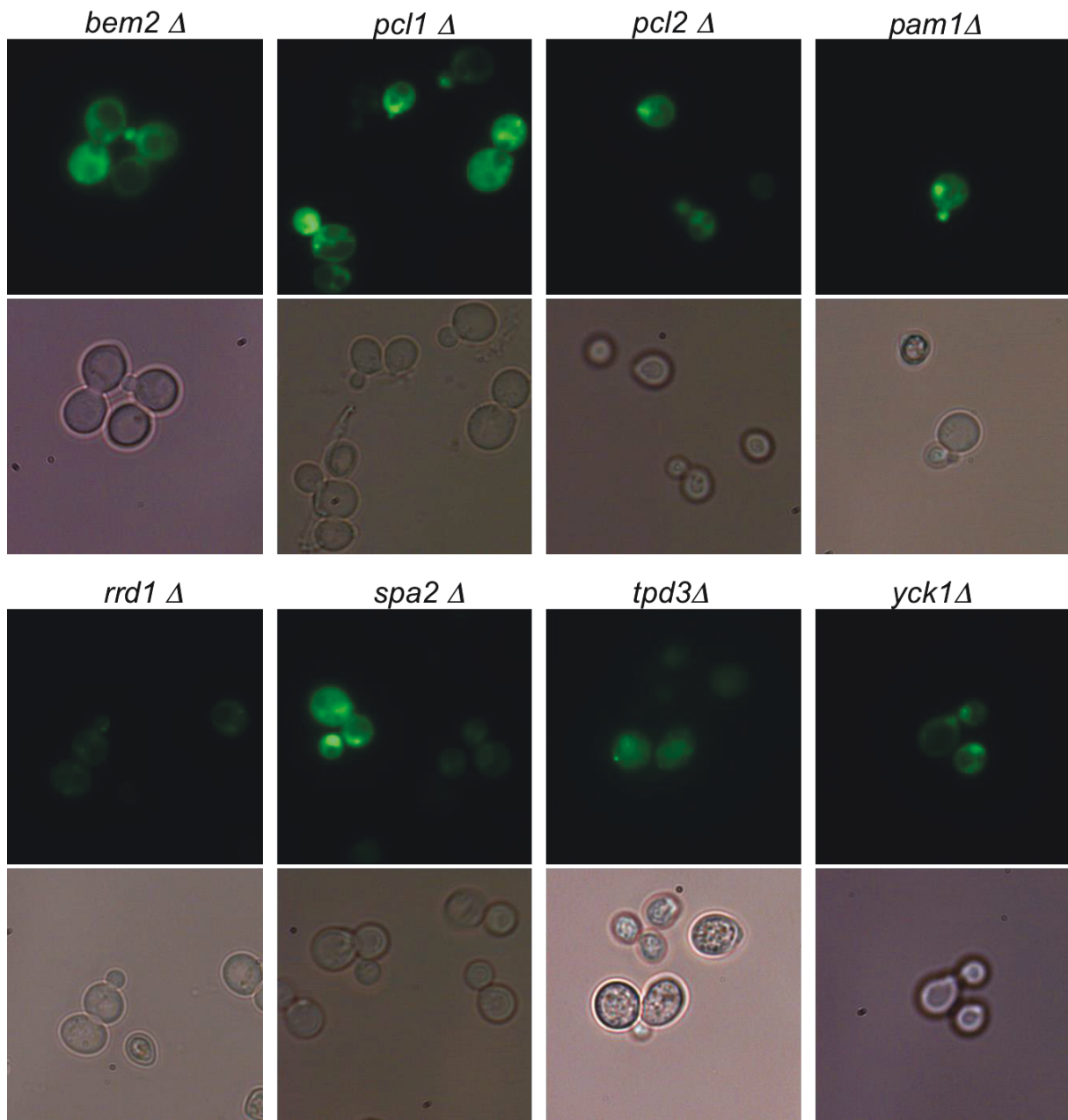


Figure 17: 8 of 20 mutants in which the localization of knr4 is abnormal

Finally, there are 3 gene deletion mutants in which the cell shape is abnormal (*ELM1*, *HSL1*, *ACE2*). Elm1 and Hsl1 proteins are involved in the bud neck formation and recruitment of other proteins at this site, but Knr4 still appears as a bright spot in these mutants (very concentrate in one point, see photographs below).

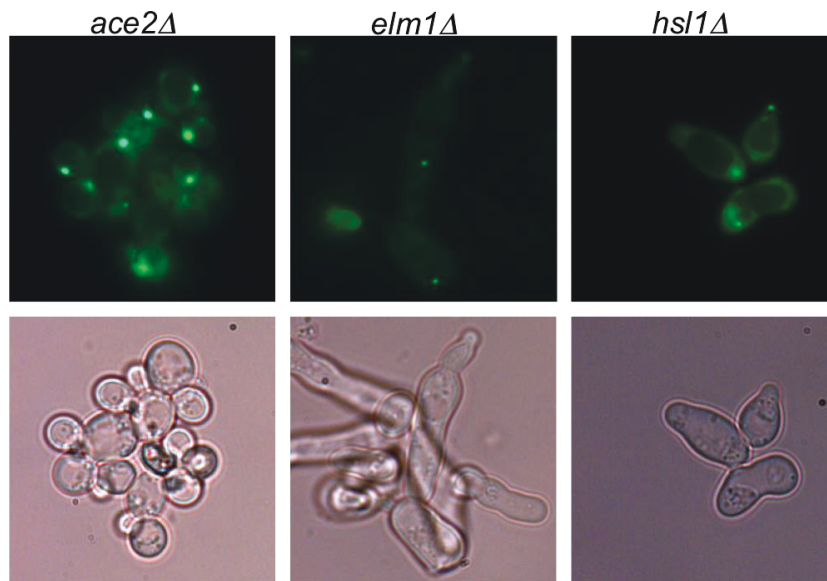


Figure 18: 3 of 20 mutants in which the cell shape is abnormal.

3.1.1.2 During the sexual differentiation, in BY4741 control strain, Knr4 localizes at the tip of the shmoo.

We used 1 hour incubation time, in YPD medium, at 30°C with 10mg/ml alpha factor (Sigma) for all mutants. In these conditions, localization of Knr4-GFP in the 20 mutants that we studied, except the mutants whose cell shape is abnormal, can also be divided into 2 groups:

- Patch concentrated at the shmoo tip (Figure 19 and Figure 20)
- Disperse in the shmoo (Figure 21).

There are 8 mutants, which appear similar to control strain BY4741 for the Knr4 localization in the tip. Also we can observe the localization in the shmoo tip but weaker than BY4741. For some mutants like the one deleted for *RRD1* gene, the intensity of GFP fluorescence is weaker than in the control strain. In deletion mutants of *SBE22* and *SBE2*, the patches of Knr4-GFP appear less concentrated than in BY4741. In all we observed that Knr4 correctly localized in most mutant (14 of 20), although its concentration appears slightly more diffuse than in BY4741 in which the Knr4 localize in one point clearly. We thus believe that the products of several of these genes involved in morphogenesis can influence the Knr4 protein localization.

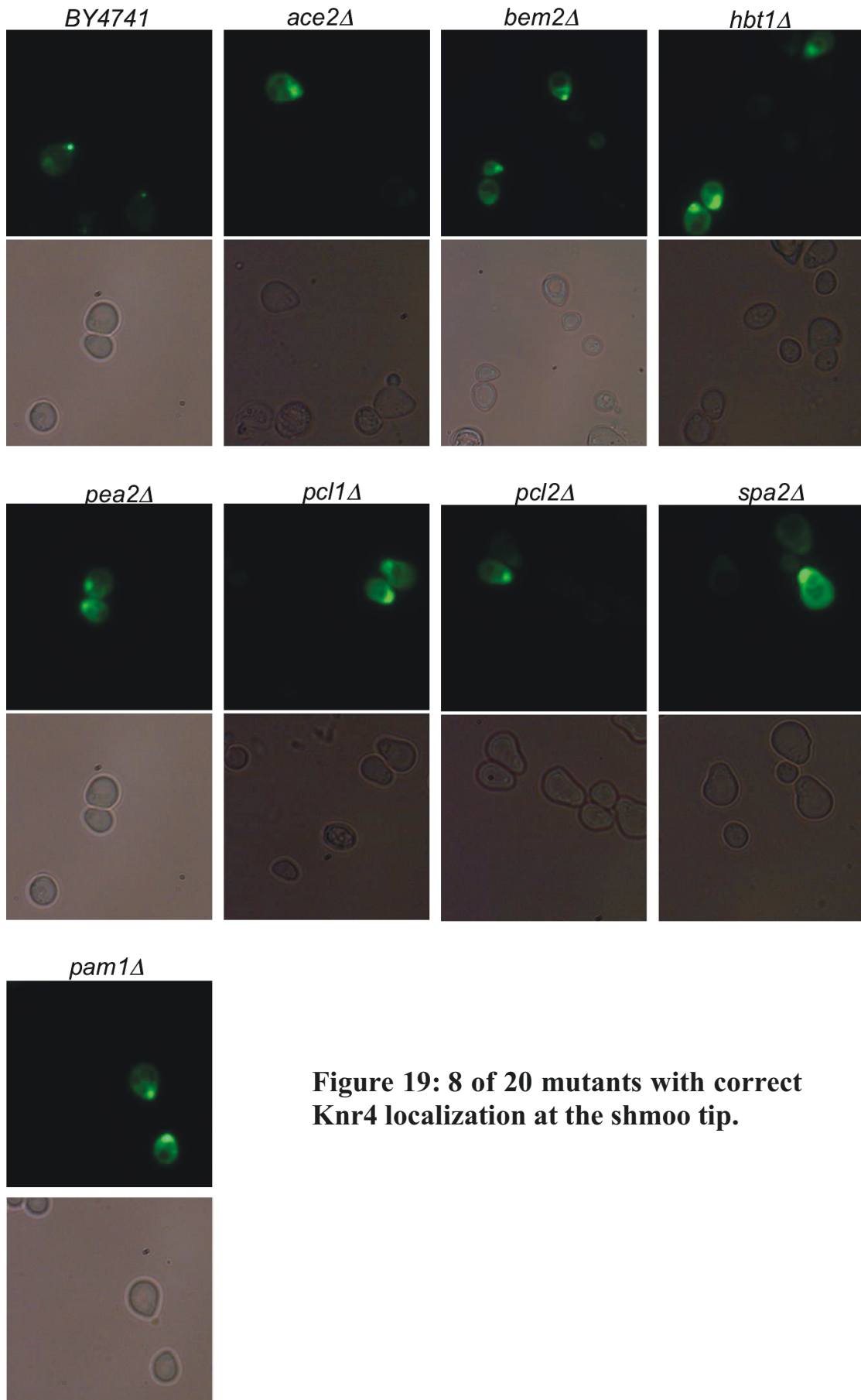


Figure 19: 8 of 20 mutants with correct Knr4 localization at the shmoo tip.

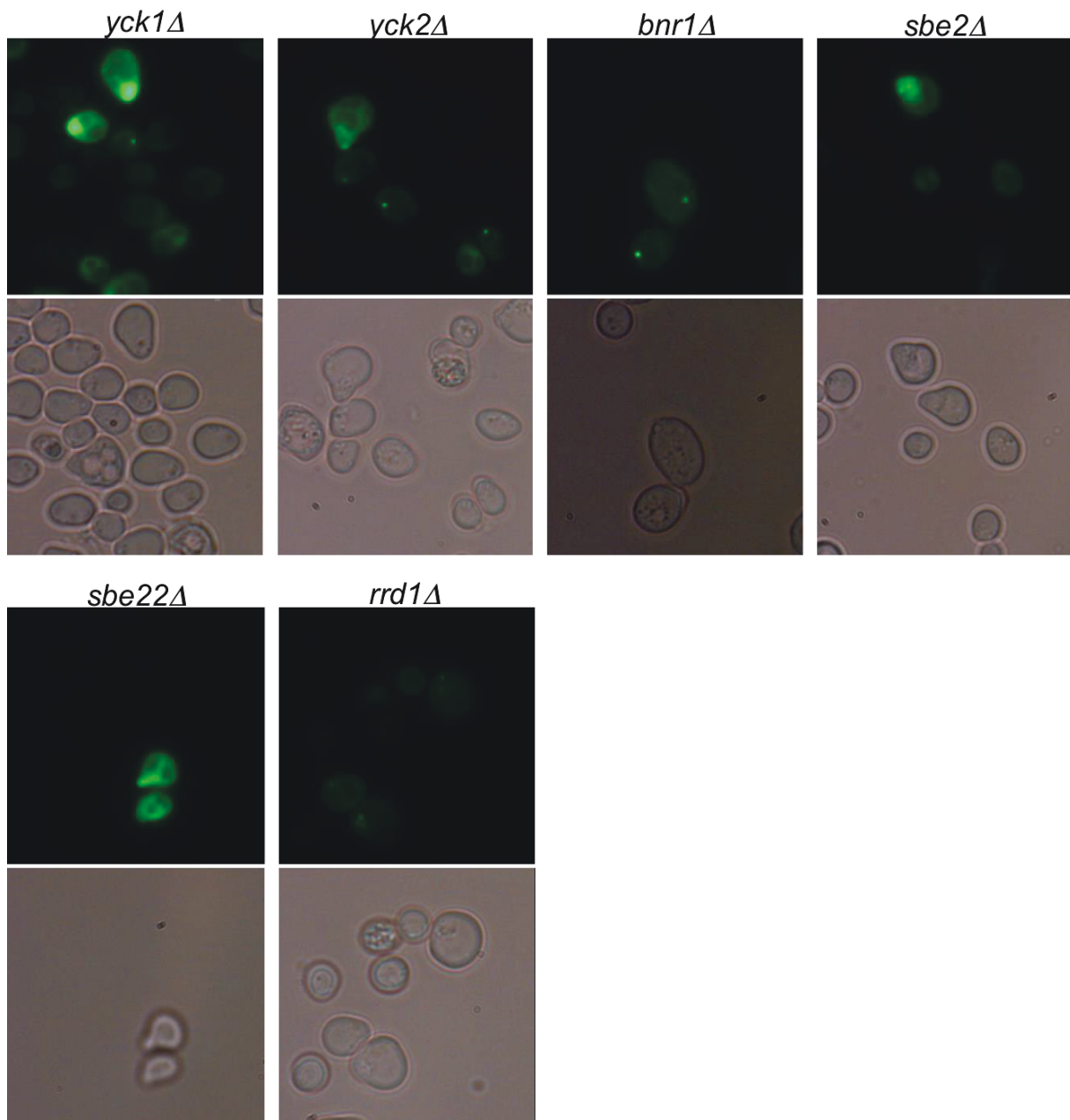


Figure 20: 6 of 20 mutants, Knr4 localization concentrated in the shmoo tip but weaker than wild type

In the mutant of *BEM1*, *BNI1*, *TPD3* and *BUD6*, the localization of Knr4 is abnormal (Figure 21). Indeed, we did not observe any spot of Knr4 in the *BEM1* and *TPD3* in cells exposed to mating pheromone. On the other hand, in mutants deleted for *BUD6* and *BNI1*, we observed a wide diffuse patch of Knr4-GFP fusion protein localized in the centre of the cells.

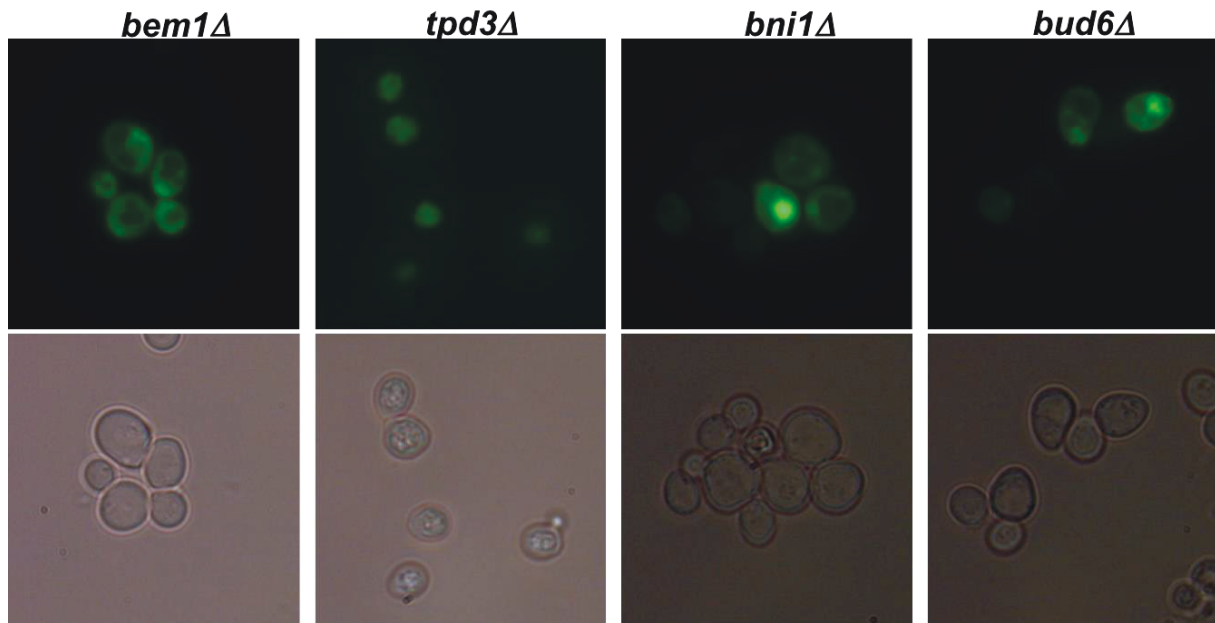


Figure 21: 4 of 20 mutants that totally lose the Knr4 localization in the shmoo

Conclusion:

- During vegetative growth (bud tip localization expected), our results show that the products of genes *BEM2*, *RRD1*, *PCL1*, *TPD3*, *PCL2*, *SPA2* and *YCK1* are required for the proper localization of Knr4.
- During sexual differentiation (shmoo tip), in the mutants deleted for genes *BEM1*, *BNI1*, *TPD3* and *BUD6* the localization of Knr4 is abnormal. It suggests these four genes products are required for the proper localization of Knr4.

In the previous experiments, Knr4-GFP was expressed from a plasmid born gene fusion. We wondered whether this might have influenced the observed localization of Knr4-GFP fusion. Hence, we decided to integrate the construct expressing Knr4-GFP in the genome, at the endogenous *KNR4* locus, under the dependence of its own promoter.

3.1.2 The localization of genome expressed Knr4-GFP is the same as from plasmid

In order to verify the cellular localization of genome expressed Knr4-GFP, we selected from the results of the previous work the 10 most interesting mutants that lose the normal localization of the Knr4 during vegetative growth (bud tip) or sexual differentiation (shmoo

tip). These 10 mutants are as follows: *BEM2*, *RRD1*, *PCL1*, *TPD3*, *PCL2*, *SPA2*, *YCK1*, *BEM1*, *BNI1* and *BUD6*.

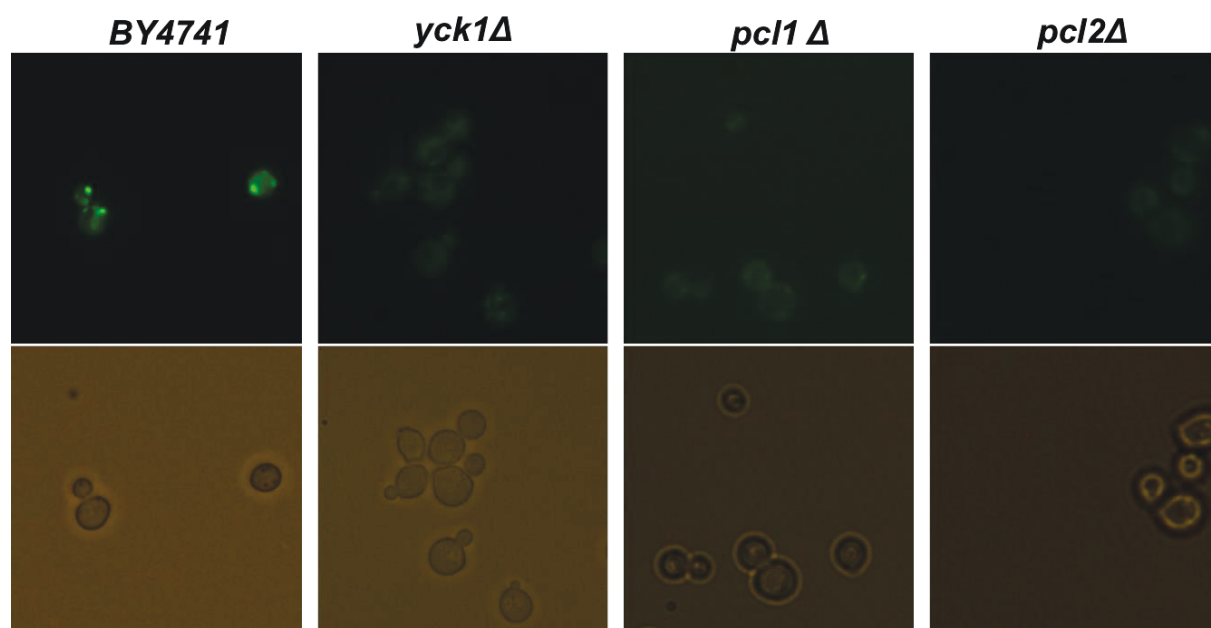
3.1.2.1 During vegetative growth

In order to ascertain the significance of our visual observations, we decided to quantify the amount of cells displaying a “normal localization” of Knr4 (i.e. like control) versus cells in which this localization is “abnormal”.

Hence, the cells from the 10 mutants in which we had the Knr4-GFP construct integrated in the genome, observed during budding, were classified into four groups according to their fluorescence pattern: bright spot in the bud tip, weaker spots in the bud tip, dispersed patches in the whole cell or no protein concentration (diffuse fluorescence in the whole cell). In the control strain BY4741, more than 50% of cells show Knr4 localized in the bud tip as a bright spot (Figure 22).

In mutants *BEM2*, *RRD1*, *PCL1*, *PCL2*, *SPA2*, *YCK1* and *TPD3* the localization of Knr4 is either absent or present as several very tiny light spots around the bud tip (in less than 10% cells).

In the mutants *BEM1*, *BNI1* and *BUD6* we can observe that the localization of Knr4 can be concentrated in some bud tips, but the ratio (10%-20%) of cells in which Knr4 correctly localizes is less than in BY4741 (Figure 22).



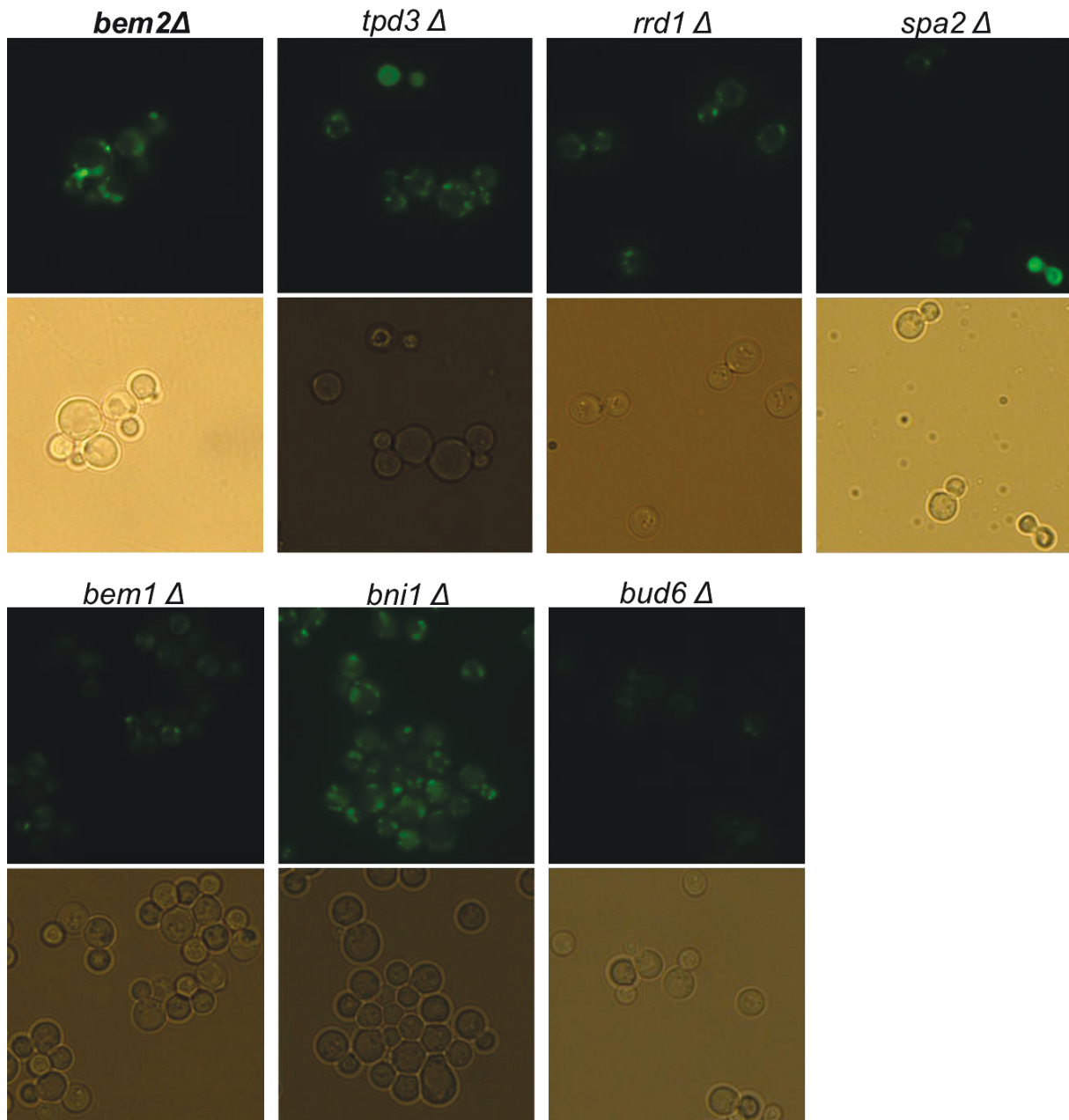
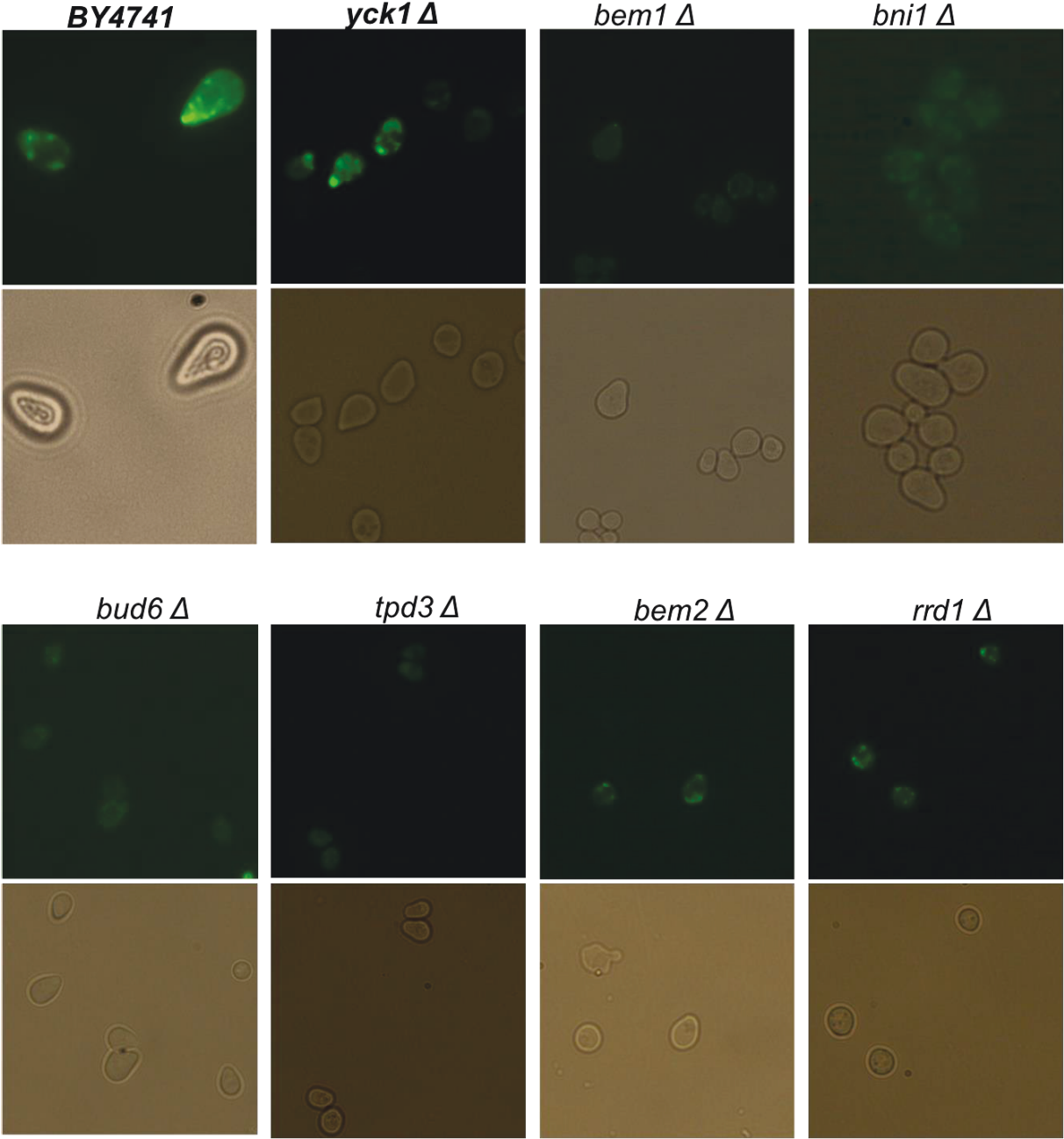


Figure 22: The Knr4-GFP localization (genome integrated construct) in the bud tip of the 10 most interesting mutants

3.1.2.2 Knr4 localization during sexual differentiation,

In these conditions, the localizations of Knr4-GFP in the 10 mutants that we studied are all abnormal in comparison to BY4741 in which Knr4-GFP concentrates in the bud tip of more than 50% cells. We observed no concentrated spots of “genome expressed” Knr4 in the mutants deleted for *BEM1*, *BEM2*, *BNI1*, *TPD3* and *BUD6*. On the other hand, in *RRD1*, *PCL1*, *PCL2* and *SPA2* deletion mutants, a weaker GFP concentration can be observed in the shmoo tip of just a few cells (less than 20%). While finally, in *YCK1* deletion mutant, we

observed the GFP concentration clearly but also just in a small amount of cells (about less than 20%).



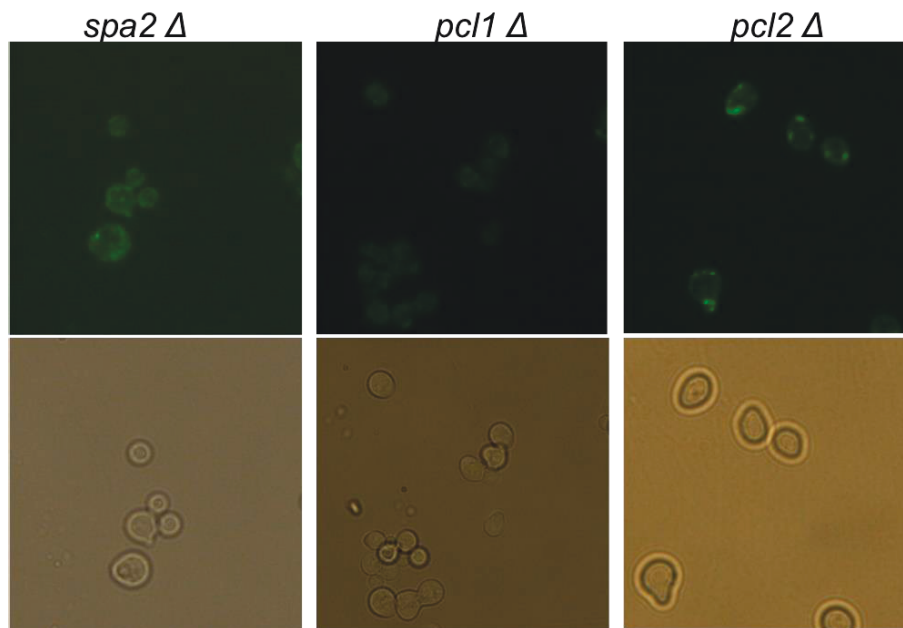


Figure 23: Localization of genome-expressed Knr4-GFP in the shmoo tip of 10 interesting mutants.

During sexual differentiation (shmoo tip), in the mutants deleted for all 10 genes we observed that the localization of genome expressed Knr4 is abnormal. As observed previously with the localization of Knr4 expressed from the plasmid, there is not any concentration of genome expressed Knr4 in the mutants deleted for *BEM1*, *BNI1*, *TPD3* and *BUD6*. It suggests these four genes products are required for the proper shmoo tip localization of Knr4. And in the other 6 genes mutants the localization of genome expressed Knr4 is also abnormal. In *BEM2*, *RRD1*, *PCL1*, *PCL2* *YCK1* and *SPA2* the GFP concentration can be observed in the shmoo tip of several cells but not as much as in BY4741. This result is similar to the result we obtained from the plasmid expressing Knr4-GFP. From the ratio of the correct localization of the cells on the total cells, we can conclude that all these 10 genes products are related to functions required for Knr4 localization during mating.

All together, our results show that there is almost no difference between the results obtained from genome integrated constructs and the ones from plasmid born constructs.

The mutants *bem2* Δ , *pcl1* Δ , *pcl2* Δ , *rrd1* Δ , *spa2* Δ , *tpd3* Δ , *bem1* Δ , *bni1* Δ , *yck1* Δ and *bud6* Δ all lose the Knr4-GFP correct localization with the *KNR4-GFP* genome integration (as with plasmid constructs).

Role of Tpd3: Our results established that the product of *TPD3* gene was needed for the Knr4 localization during both vegetative growth and sexual differentiation. **Tpd3** is the Regulatory

subunit A of the heterotrimeric protein phosphatase 2A (PP2A), which also contains regulatory subunit Cdc55p and either catalytic subunit Pph21p or Pph22p; required for cell morphogenesis and transcription by RNA polymerase III. Hence, to follow up on these results, we decided to check the Knr4p localization in the mutant deleted for genes *CDC55*, *PPH21* and *PPH22*. It may also be interesting to test the activation state of CWI and Calcineurin pathways in these mutants, for example by measuring the transcription from Crz1 or Rlm1 activity reporter plasmids.

3.1.3 The localization of Knr4 in mutants *pph21* or *pph22* and *cdc55*

We transformed mutants *pph21* or *pph22* and *cdc55* by plasmid pHM43 bearing the Knr4-GFP protein fusion construct. We verified whether the cellular localization of Knr4 was conserved in these mutants during vegetative growth (among the cell cycle especially) and sexual differentiation.

During vegetative growth (bud tip localization expected), the localization of Knr4 in mutants *pph21* or *pph22* and *cdc55* is normal, similar to the one observed in BY4741. (Figure 24)

During sexual differentiation (shmoo tip), Knr4 in the mutants *cdc55* and *pph22* localize at the shmoo tip which is normal. However, in the mutant *pph21* Knr4 localization in the shmoo is significantly more diffuse (no clear bright spot). Pph21 and Tpd3 are both part of the protein phosphatase 2A (PP2A) complex. So PP2A function, including catalytic function of Pph21, may be implicated in events required for the correct localization of Knr4 at the shmoo tip (see Figure 24). Remarkably, studies on Tpd3 protein localization during the cell cycle and during shmoo formation have shown that it is located at the tip of small buds and at the shmoo tip as *knr4*, and that this localization is dependant on the presence of the catalytic PP2A subunits Pph21 and Pph22 (Gentry M. S., Hallberg R. L., Mol Biol Cell., 14, 3477-3492).

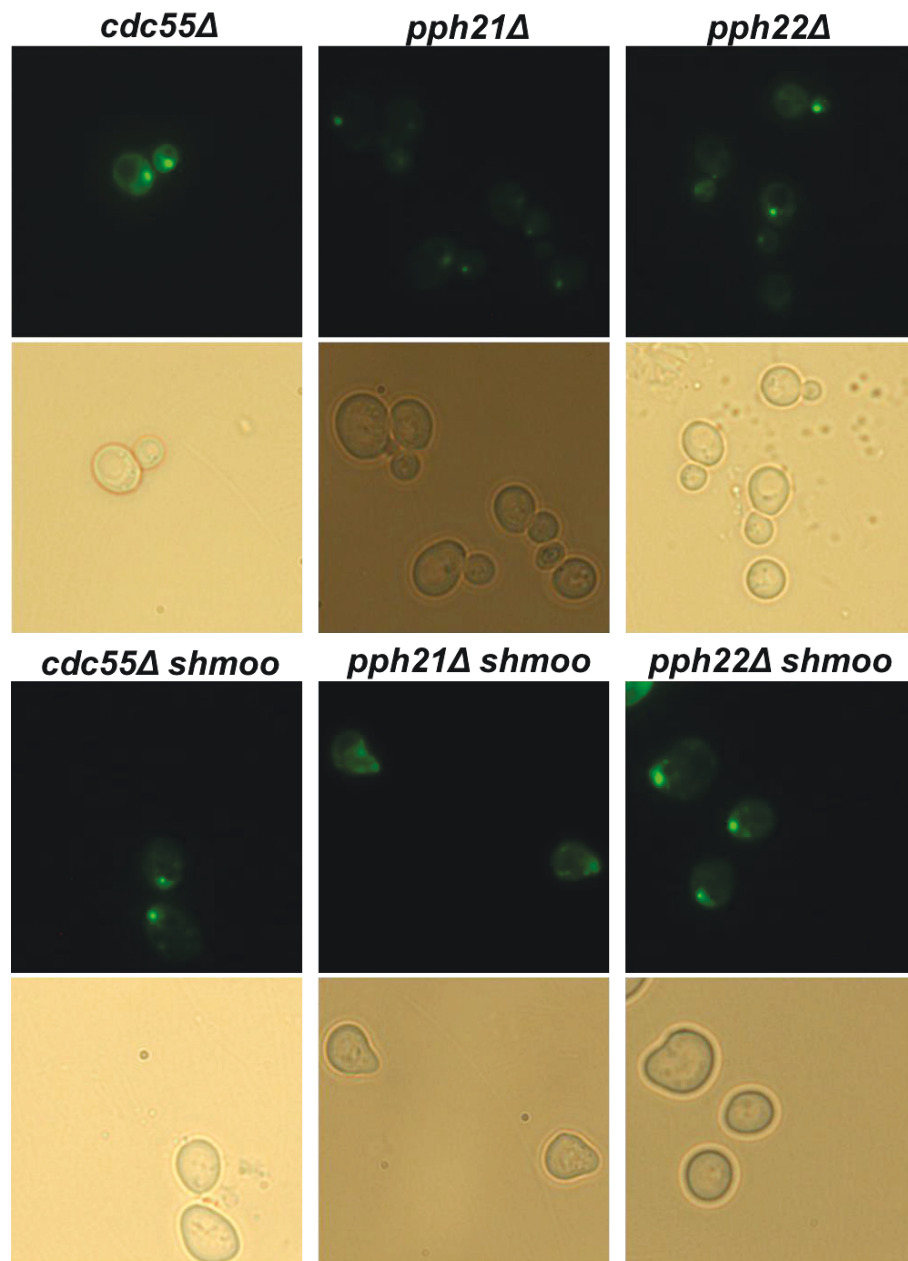


Figure 24 : Localization of Knr4-GFP in mutants *pph21*, *pph22* and *cdc55* during vegetative growth and sexual differentiation

3.1.4 Are the calcineurin pathway and CWI pathway involved in the correct localization of the Knr4?

The links between *KNR4* and the PKC1 (or CWI) pathway have been investigated already (Martin-Yken et al., 2003). The physical interaction between Knr4 and the MAP kinase Slt2 has been demonstrated by several complementary methods. This interaction is proposed to modulate Slt2 activation of transcriptional factors Rlm1 and SBF (Martin-Yken et al, 2003).

In addition, the presence of Knr4 protein in the cell notably with its N-terminal domain which is necessary for the correct Knr4 localization, is absolutely necessary for cell viability in the absence of a functional Pkc1-dependent cell integrity pathway (Durand et al. 2008; Dagkessamanskaia, et al. 2010). So we raised the following question: is Sl2, which interacts with Knr4, necessary for Knr4 correct localization? To answer this, we introduced the Knr4-GFP (plasmid) into the *slt2Δ* mutants and to observe the localization of Knr4 during vegetative growth and shmoo formation. Our results show that the localization of the Knr4 in the bud is normal and in the shmoo tip as well (long time 2h incubation with 15μg/ml α-factor get 20% shmoo in *slt2Δ*.) So the Sl2 is not necessary for Knr4 localization.

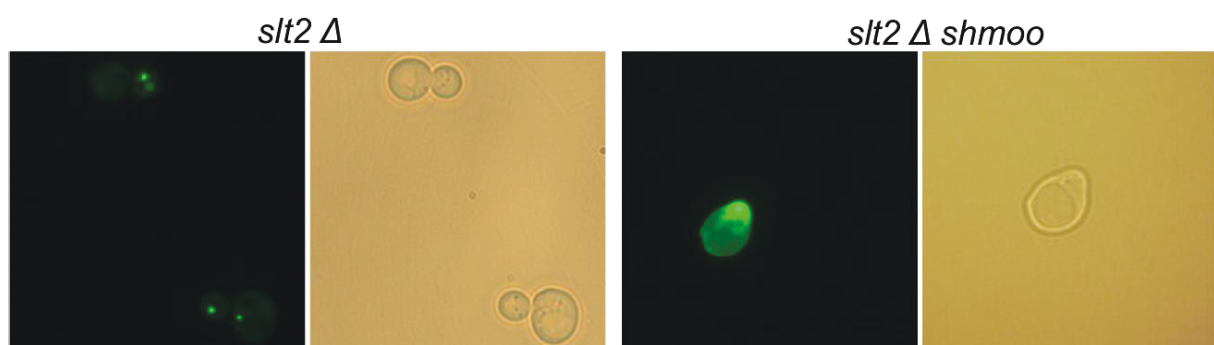


Figure 25: Knr4 localization in the *sltΔ* mutants

Calcineurin is a heterodimer consisting of two subunits, the α -catalytic subunit, termed calcineurin A, which is about 60 kDa, and binds to Ca^{2+} , and a regulatory β -subunit, termed calcineurin B which is about 19 kDa. Protein-protein interactions studies in the two hybrid system between Knr4 and these two different subunits of Calcineurin revealed no significant interaction with the regulatory sub-unit Cnb1, while a strong interaction was detected between Knr4 and the catalytic subunit Cna1 (Dagkessamanskaia, et al. 2010). So we tested the Knr4 localization in the *cna1Δ* mutant. Our results showed that the localization in the bud is normal as in BY4741. But interestingly, during shmoo formation, we could only see the correct Knr4 localization in very little cells (less than 15%), so the Knr4 localization during mating seems affected by the absence of the Cna1 (See figure 26). The links between Knr4, calcineurin and the polarized growth organization are very appealing targets for follow up of this work.

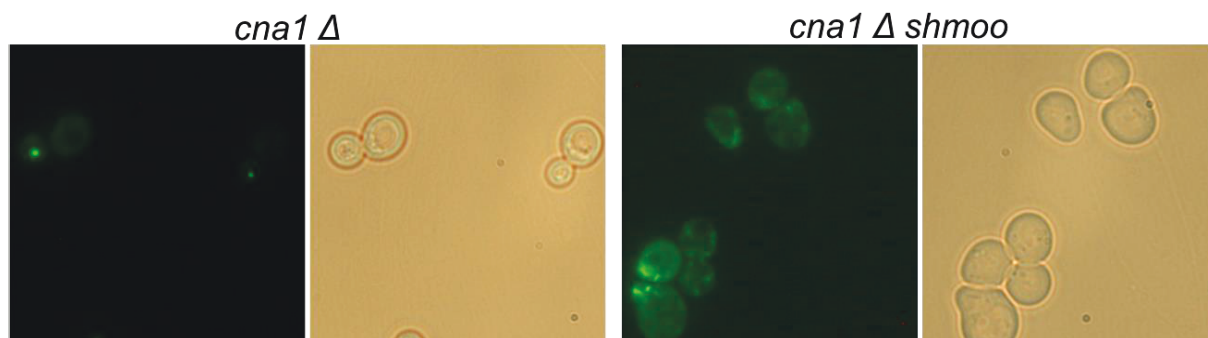


Figure 26: Knr4 localization in the *cna1*Δ mutants

3.1.5 Conclusion and Discussion

For the localization of the Knr4, we observed GFP-Knr4 (expressed from either plasmid or genome integration) localization in the bud and shmoo of 25 mutants. First, we tested Knr4 localization in 20 mutants related to the “Morphogenesis” and “Cell Polarity”. 10 of 20 mutants have been found to display an abnormal Knr4 localization in the bud or shmoo. This result has been confirmed by the observation of the localization in the mutants bearing genome integrated Knr4-GFP construct. These ten mutants are the ones deleted in the genes BEM2, RRD1, PCL1, TPD3, PCL2, SPA2, YCK1, BEM1, BNI1 and BUD6. In these mutants, Knr4 protein is either not visible or localized in different places during the cell cycle (Figure 27). The majority of these genes are involved in the polarized growth during the yeast cell cycle. It should be noted that the products of almost all them have been reported to localize at the presumptive bud site in G1, at the tip of small buds or at the mother-daughter neck during cytokinesis, as Knr4 protein (Figure 27). Pcl1, Pcl2, Bem1, Bem2, Tpd3, Spa2, Bud6 and Bni1 with 2 additional partners Pph21 and Cna1, which are necessary for the correct Knr4 localization, display themselves a similar cellular localization as Knr4.

We conclude our 12 Knr4 partners, which required for the Knr4 localization into three groups by their function. First group, which is related to the establishment of cell polarity, contains Pcl1, Pcl2, Bem1, Bem2, Bni1, Bud6 and Spa2. The second group relates the function of phosphatases (PP2A and Calcineurin) and contains Tpd3, Rrd1, Pph21, Yck1 and Cna1.

Pcl1 and Pcl2: Pcl1p and Pcl2p are cyclins that interact with the cyclin-dependent kinase (CDK) Pho85p (Huang et al. 2007). They belong to the Pcl1,2 subfamily of cyclins, which are primarily required for progression through the cell cycle and regulating cell polarity and

morphogenesis (Huang et al. 2007; Measday et al. 1997). PCL1 and PCL2 ensure phosphorylation of key regulators of polarized growth, septin ring assembly, and morphogenesis in G1 (Egelhofer et al. 2008; Zou et al. 2009).

Pcl1p and Pcl2p have some redundancy with Cln1p and Cln2p, two G1-cyclins that interact with CDK Cdc28p (Huang et al. 2007; Carroll & O'Shea 2002). Corresponding to their role in G1, PCL1 and PCL2 expression and activity peak in G1 (Measday et al. 1997; Measday et al. 1994). Consistent with their role in regulating morphogenesis and polarized growth, Pcl1p and Pcl2p are localized at the bud neck and sites of polarized growth, where are the Knr4 localization point (Moffat & Andrews 2004). And our results also show that without the Plc1 and Pcl2 the Knr4 localization is abnormal. So Pcl1 and Pcl2 are important for the Knr4 localization and function in morphogenesis.

Bem1 helps to establish the cellular polarity that is required for bud and shmoo formation. An excellent review written by Madden and Snyder describes the process of establishing cell polarity and morphogenesis in yeast (Madden & Snyder 1998). Bem1p is an SH3-domain protein that binds Cdc24p, which is a nucleotide exchange factor for Cdc42p, a Rac/Rho GTPase. Because Bem1p also binds Ste5p and Ste20p, which are central components of the mating pathway, the role of Bem1 may be to connect the mating signal to the proteins that induce the appropriate changes to the actin cytoskeleton (Leeuw et al. 1995). Our result shows that without Bem1, Knr4 can't localize in the shmoo tip, which is its correct localization. So we think Knr4 function may be related to the actin cytoskeleton through the Bem1 protein. Bem1p, Cdc24p, and Cdc42p also interact with a GTPase complex that is involved in budding (Johnson & Pringle 1990; Zheng et al. 1993). This GTPase complex contain a Ras-related GTPase, whose function may be to localize Bem1p, Cdc24p, and Cdc42p at the nascent bud site where they can reorganize the actin cytoskeleton to establish polarization and where Knr4 localize (Park et al. 1997).

Bem2 is a Rho GTPase activating protein (RhoGAP) involved in the control of cytoskeleton organization and cellular morphogenesis. It is also required for bud emergence (Wang & Bretscher 1995; Kim et al. 1994). Our results just show that the localization of the Knr4 in the budding yeast is dependant on Bem2. It is possible that Knr4 is involed in the morphogenesis and cytoskeleton by the interaction with this protein.

Polarisome complex is formed by Pea2, **Bni1**, **Bud6**, and **Spa2** localized to sites of polarized growth (Fujiwara et al. 1998; Sheu et al. 1998)(Figure 27). It is thought to form a

link between polarity establishment factors and actin cables. And Bni1, Bud6 and Spa2 are all required for the Knr4 localization. Thus, it is relatively safe to conclude that the proper cellular localization of Knr4 among the cell cycle depends on the Polarisome complex, as it is the case for several other morphogenesis related proteins.

Tpd3 is the Regulatory subunit A of the heterotrimeric protein phosphatase 2A (PP2A), which also contains regulatory subunit Cdc55p and either catalytic subunit Pph21p or Pph22p. It is required for cell morphogenesis and transcription by RNA polymerase III (Wang & Burke 1997; van Zyl et al. 1992). Our results show that Tpd3 is required for the localization of the Knr4 during both budding and shmoo formation. But among the other three subunits Cdc55p, Pph21p and Pph22p, only Pph21 is required for the localization of the Knr4 in the shmoo tips. **Rrd1**, which is required for the Knr4 localization is an activator of the PP2A. Suppression of the lethal phenotype of the *rrd1,2* Δ by overexpression of *PPH22* suggest that the Rrd1 functions positively towards the catalytic subunits of PP2A (Rempola et al. 2000).

Yck1 and Yck2 share redundant functions with each other in morphogenesis, proper septin assembly, and endocytic trafficking. They provide an essential function overlapping with each other. They are also activators of the phosphotyrosyl phosphatase activity of PP2A. They are involved in G1 phase progression, microtubule dynamics, bud morphogenesis and DNA repair (Robinson et al. 1993; Robinson et al. 1999; Roth et al. 2002; Babu et al. 2004). Our results show correct Knr4 localization needs the Yck1. All together, our results suggest that PP2A function is involved in the cellular localization of Knr4 through Rrd1, Tpd3, Pph21, and Yck1. However, whether Knr4 physically interacts with any me members of the complex or even is a direct substrate of PP2A remains to be determined.

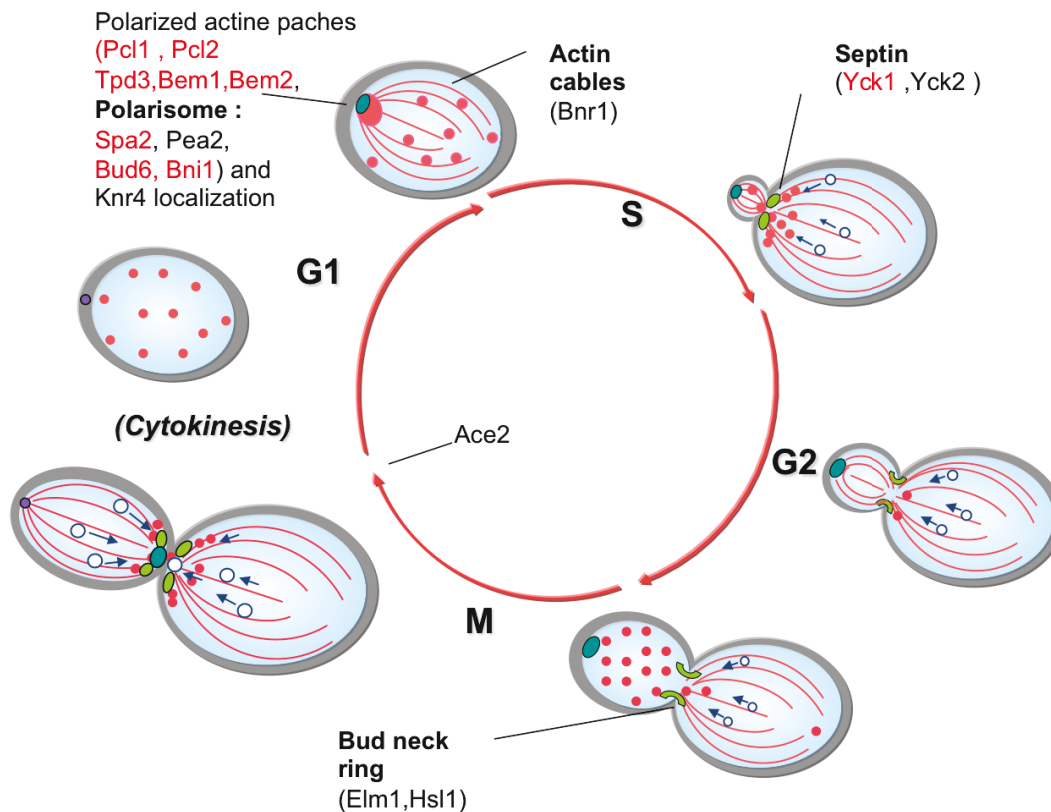


Figure 27 The localization of proteins during the cell cycle of yeast

The cell cycle of budding yeast commitment in late G1 phase triggers the assembly of the polarized patch (pink) and septin ring (green), as well as the polarization of actin cables and cortical actin patches. Gene Protein names in red are the interesting genes related to the Knr4 localization.

KNR4 gene deletion can cause synthetic lethality with CWI pathway and calcineurine pathway (Durand et al. 2008). Slt2, the final effector of the CWI MAP kinase cascade, does not influence the localization of Knr4. But there is a study showing that introduction of *slt2Δ* into the *rrd1,2Δ* background improved the growth of *rrd1,2Δ* on sorbitol-containing medium, indicating some interaction between Rrd1 and the CWI pathway (Rempola et al. 2000). Our results suggest that Knr4 might constitute a new link between Rrd1 and the CWI pathway signalling. But the relation between the Knr4 with Rrd1 and Slt2 / CWI pathway obviously requires further study.

In addition, we found out that Cna1, the catalytic subunit of Calcineurin, another key phosphatase involved in cell signalling, is also required for the Knr4 correct localization. The important role of phosphatases (Calcineurin and PP2A) in the cellular localization of Knr4

protein prompted us to directly check for a possible role of the known *in vivo* phosphorylation sites of Knr4.

3.2 The function of the Phosphorylation sites and the fragments of the Knr4

Post-translational modifications (PTMs) of proteins are indispensable elements of the semantics of cellular communication. PTMs are reversible, enzymatically-mediated modifications of specific amino acid side chains. Some common types of PTMs include phosphorylation and O-glycosylation on Ser (S) and Thr (T) residues, phosphorylation on Tyr (Y) residues, methylation on Arg (R) residues, and acetylation and ubiquitination on Lys (K) residues. Protein phosphorylation describes the attachment of a phosphate group onto a specific residue of the protein, usually a serine, threonine or tyrosine or, often more transiently, on histidine residues. Phosphorylation has the remarkable ability to turn on and off the function of many enzymes, thereby altering their activity, interactions with their partners or substrate and sometimes their cellular localization.

Reversible phosphorylation of proteins is an important regulatory mechanism that occurs in both prokaryotic and eukaryotic organisms. Kinases phosphorylate proteins and phosphatases dephosphorylate proteins. Many enzymes and receptors are switched "on" or "off" by phosphorylation and dephosphorylation. Reversible phosphorylation often results in a conformational change in the structure of enzymes and receptors, causing them to become activated or deactivated

Given the involvement of Knr4 in the PKC1 pathway (a kinase cascade) and calcineurin pathway (a phosphatase relay), we figured that Knr4 potential protein phosphorylation might be a relevant question. Accordingly, global studies of the yeast phosphoproteome have revealed 18 *in vivo* phosphorylated residues in Knr4 protein. All these 18-phosphorylated residues are located on the central core and C-terminal of Knr4. Comparing to the C-terminal, which has negative effects for its interactions with the partners, the central core of the Knr4 (AA 80 to 340) is considered as the functional part. This central core alone is sufficient to complement most of the *knr4* mutant phenotypes. It is also able to rescue synthetic lethality of the double deletions *rlm1Δknr4Δ* and *swi4Δknr4Δ*. Knr4 central domain is necessary for Slt2 MAP kinase to activate Rlm1, SBF and the others transcriptional factors and thus to maintain cell viability in the absence of one of them. Serine 200 and serine 203; located in the central core of the Knr4, have been proposed to be phosphorylated by another member of the CWI pathway, MKK1 which is the MAP kinase kinase responsible for Slt2 *in vivo* phosphorylation

(ref). An attempt to mimick constitutive dephosphorylated state by substitution of these two serine residues by alanines resulted in loss of the physical interaction with Tys1 (Fadi Basmaji et al. 2006). We wanted to further investigate the function of these two phosphorylated residue, so we used aspartic acid (D) to substitute the serines acid in order to imitate the constitutive phosphorylated state of these two serine residues (Figure 28). At last, we had two distinct phosphorylation mutants on Serine200 and Serine203: $\text{Knr4}^{\text{S200AS203A}}$ that mimics the dephosphorylated state, and $\text{Knr4}^{\text{S200DS203D}}$ that mimics the phosphorylated state (Figure 28). These two mutants were then used as follows for investing the function of these two residues.

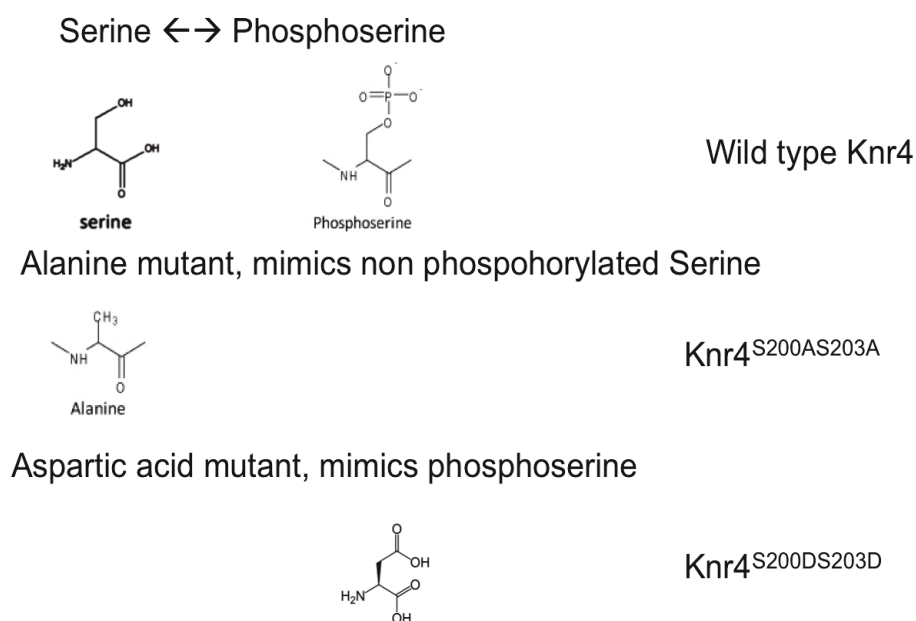


Figure 28: Structural basis for the choice of AA used to mimic phosphorylated and dephosphorylated states of serine residues

3.2.1 Two-hybrid test for $\text{Knr4}^{\text{S200D203D}}$ with Cna1 and Tys1

We tested $\text{Knr4}^{\text{S200D203D}}$, Knr4 and $\text{Knr4}^{\text{S200A203A}}$ interaction with Cna1 or Tys1 in the Two-hybrids system. For this test, we observed growth on minimal medium Ade- and His-, and we measured the beta-galactosidase activity.

From the results of the ADE2 reporter gene, which is known to be the most stringent of all, we can get that both mutants $\text{Knr4}^{\text{S200D203D}}$ and $\text{Knr4}^{\text{S200A203A}}$ with Tys1 and Cna1 did not grow. And from the results of the His reporter, which is less stringent, the mutants Knr4

S200D203D and Knr4^{S200A203A} with Tys1 and Cna1 do not grow well also but Knr4^{S200D203D} grows a little better than Knr4^{S200A203A}. (Fig 29 and Table 6)

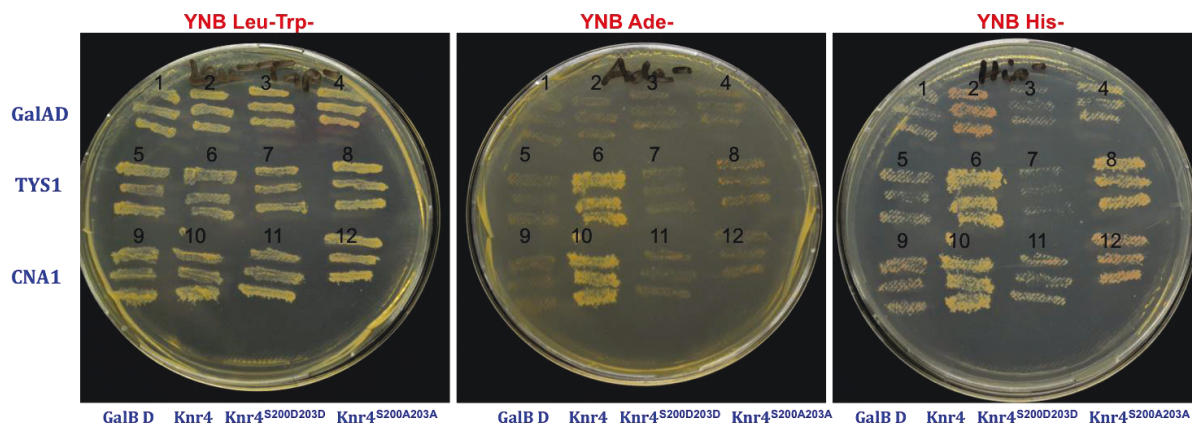


Figure 29: Two hybrid results on the plate

Table 6: β -galactosidase activity and growing results on plate of two hybrid system

N°	Plasmid contained	Grow on plate Ade-	Grow on plate His-	β -galactosidase activity (nml /min/mg of protein)
1	PGAD424 & POBD-80	-	+	30,50
2	PGAD424 & POBD-80-Knr4-WT	-	++	53,48
3	PGAD424 & POBD-80-Knr4-S200AS203A	-	-	32,43
4	PGAD424 & POBD-80-Knr4-S200DS203D	-	+	19,55
5	PGAD-TYS1& POBD-80	-	+	24,07
6	PGAD-TYS1& POBD-80-Knr4-WT	+++	+++	88,51
7	PGAD-TYS1&	-	-	41,32

	POBD-80-Knr4-S200AS203A			
8	PGAD-TYS1&POBD-80-Knr4-S200DS203D	-	++	35,22
9	PGAD-CNA1&POBD-80	-	+	26,17
10	PGAD-CNA1&POBD-80-Knr4-WT	+++	+++	60,73
11	PGAD-CNA1&POBD-80-Knr4-S200AS203A	-	+	31,73
12	PGAD-CNA1&POBD-80-Knr4-S200DS203D	-	++	29,16

Results of the beta-galactosidase activity dosage showed that:

- Interaction strength of Knr4^{S200D203D} (35.22/ β -galactosidase activity) with Tys1 is similar with Knr4^{S200A203A} (41.32/ β -galactosidase activity both are significantly lower than the Knr4 wild type (88.51/ β -galactosidase activity) and higher than empty plasmid (24.07/ β -galactosidase activity).

- Interaction strength of Knr4^{S200D203D} (29,16/ β -galactosidase activity) with Cna1 is also similar to Knr4^{S200A203A} (31.73/ β -galactosidase activity). The interaction strength of the two mutants is lower than wild type Knr4 (60.73/ β -galactosidase activity) and very close to the negative control with empty plasmid (26.17/ β -galactosidase activity).

All these results suggest that the phosphorylation sites of the residues S200 and S203 can influence the protein interaction with Tys1, as reported before (Basmaji *et al.*, 2006), and also influences the interaction with the Cna1.

3.2.2 The phosphorylation sites S200 and S203 of the Knr4 do not influence the localization of Knr4

Knr4 is known to be a regulatory protein, which participates in the coordination of cell wall synthesis with bud emergence. We wanted to check whether this function was related and/or possibly controlled by *in vivo* phosphorylation of this protein. The results exposed here above show that the phosphorylation sites S200 and S203 influence Knr4 interactions with its protein partners, and the research published before showed that the Knr4 mutant S200A203A also led to a weaker complementation of *knr4* null mutant phenotypes. (Basmaji et al, 2006) . In order to first test the influence of these two *in vivo* phosphorylation sites on the cellular localization of Knr4 during the cell cycle, we constructed the plasmids expressing Knr4S200A203A and Knr4S200D203D with GFP (pUG35-Knr4 S200A203A-GFP, pUG35-Knr4 S200D203D -GFP), transformed them in the Knr4 null mutant and BY4741 control strain, and observed the transformants under fluorescence microscopy. pHM43 (pUG35-Knr4-GFP) was used as control for the correct protein localization. Since we have shown that the localization of the GFP fusion constructs on this plasmid is the same as the one observed from genome integration, we think that this is correct to use this plasmid.

Here are the results of the localization during the vegetative growth and shmoo formation:

Knr4 and Knr4 phosphorylation sites mutants in *knr4* mutants and BY4741 are all localized in the bud tip during vegetative growth and in the shmoo tip during sexual differentiation (Figure 30 and Figure 31). So we conclude that the phosphorylated status of sites S200 and S203 do not significantly influence Knr4 localization, as far as can be deduced from our amino acids substitution strategy.

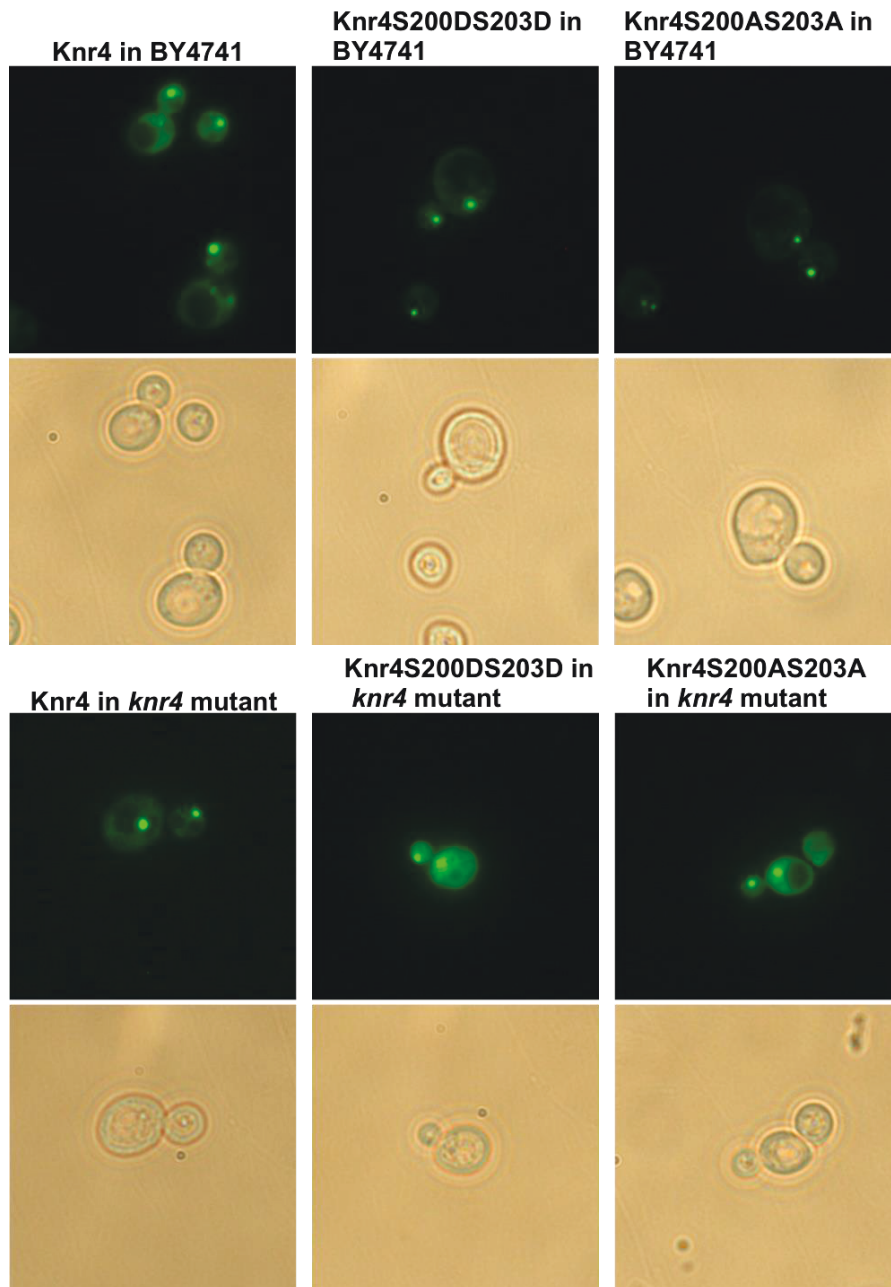


Figure 30 The localization of the Knr4 phosphorylation sites mutant in the *knr4Δ* and BY4741

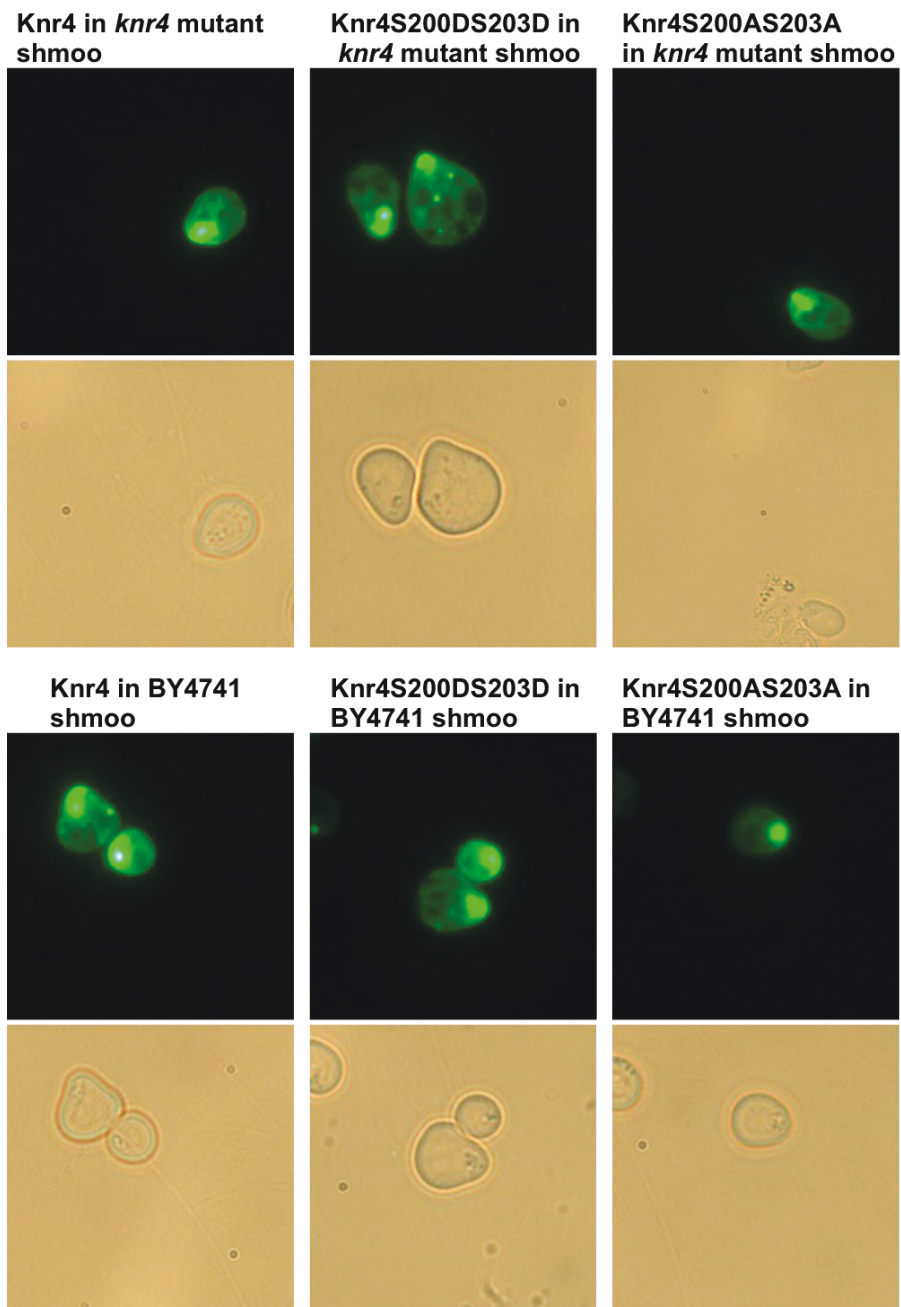


Figure 31 The localization of the Knr4 phosphorylation sites mutants in the shmoo of *knr4Δ* and BY4741

3.2.3 The phosphorylation sites of the Knr4 might play a role in the CWI pathway

3.2.3.1 The serine 200 and 203 of Knr4 are involved in the CWI pathway by Mkk1

It has been known that Knr4 is involved in the CWI pathway, and more specifically, that Knr4 is required for Slt2 to accurately activate its downstream targets, transcription factors Rlm1 and SBF (Martin-Yken et al., 2003). In order to test the function of the phosphorylation sites S200 and S203 in relation with the CWI pathway, we performed assays of growth rescue of synthetic lethality. *KNR4* gene deletion combined with additional deletion of several yeast genes, including *BCK1* or *SLT2*, results in synthetic lethality. We used these two double mutants (*bck1Δknr4Δ* & *slt2Δknr4Δ*) transformed by plasmid pHM37 expressing the protein Knr4 to ensure yeast viability. Plasmid pHM37 has URA3 marker gene that encodes Orotidine 5'-phosphate decarboxylase (ODCase). This plasmid can be selected for on Ura-selective medium. In contrast, if 5-FOA (5-Fluoroorotic acid) is added to the media, the active ODCase will convert 5-FOA into a toxic compound (a “suicide inhibitor”) 5-fluorouracil causing cell death, which allows for selection against yeast carrying the gene (Fabien Durand thesis 2008).

So we transformed individually pHM38, pFB1, pRS316- $KNR4^{S200D203D}$ and empty pRS316 plasmid, which express respectively wt Knr4 protein, $KNR4^{S200AS203A}$, $KNR4^{S200D203D}$ and no *knr4* protein at all, into *bck1Δknr4Δ* and *slt2Δknr4Δ*. After double selection on Leu- Ura-, we replicated the strains on 5-FOA plates in order to negatively select for the loss of pHM37.

We already know that the empty plasmid cannot rescue these two mutants as does the wild type Knr4 protein. Our new results show us that both two Knr4 phosphorylation sites mutants cannot rescue the *bck1Δknr4Δ*, while both two mutants can rescue *slt2Δknr4Δ* (Figure 32). So the ability of Knr4 protein to be phosphorylated or dephosphorylated on at least one of the serine residues 200 and 203 is required for the bypass of the upstream part of the CWI pathway, such as signal transmission from Pck1 to Bck1 or Bck1 to Mkk1/ Mkk2. While on the opposite, this ability of Knr4 to be phosphorylated or dephosphorylated on these two sites is not required for the bypass of the end of the CWI pathway, namely the signal transmission from Mkk1/Mkk2 to Slt2 or from Slt2 to its targets.

Global studies have raised the possibility that residues S200 and S203 might be phosphorylated *in vivo* by Mkk1 (<http://www.phosphopep.org/>). However, since Serine 200

and Serin 203 residues of Knr4 are in fact not required for the rescue by Knr4 of the synthetic lethality of *slt2Δknr4Δ*, we believe that if there is a signal transmission from Mkk1 to Knr4 and further to cell rescue in the absence of Slr2, then it has to take place through other residues of Knr4. This parallel pathway ensuring cell survival in the absence of a functional CWI pathway can be the Calcineurin pathway.

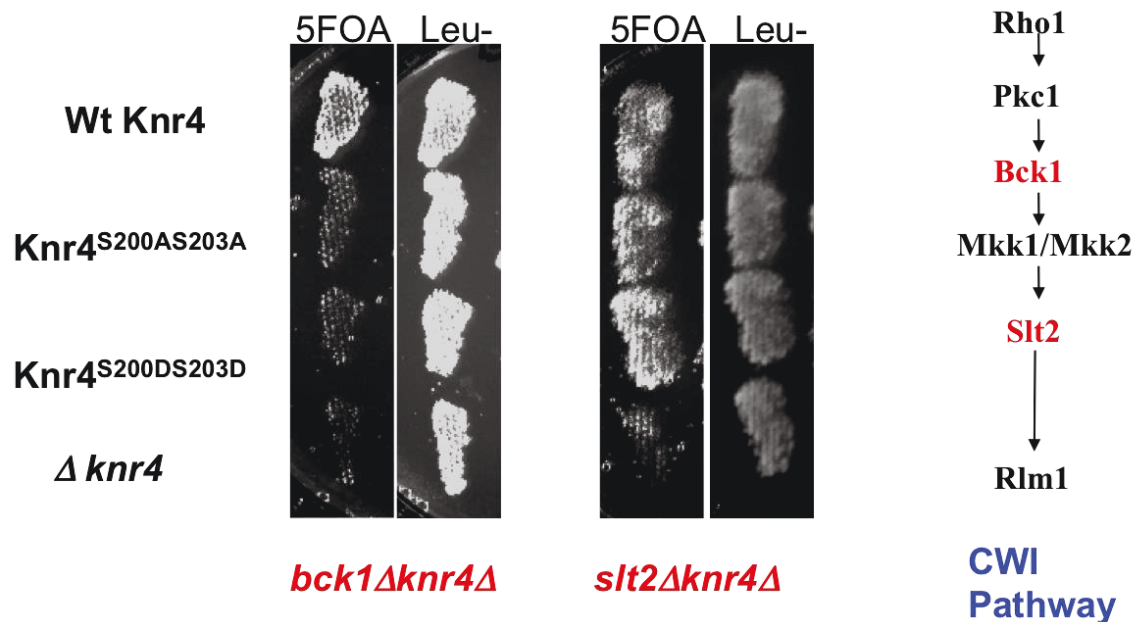


Figure 32 Assay of growth rescue of synthetic lethality between *knr4Δ* and CWI pathway members

In order to investigate further the function of the S200 and S203 related to CWI signalling and Mkk1, we took advantage of the plasmid p1025-*MKK1-P386* which encodes a constitutively activated mutant of Mkk1. Expression of this mutant allele Mkk1-P386 on galactose medium results in cell growth inhibition. This growth-inhibitory effect is suppressed by the *slt2Δ* mutation, suggesting that it is indeed the hyperactivation of the CWI pathway, which is toxic to cells. To test the function of the Knr4 that interacts with the Mpk1 pathway, we transformed the p1025 into the genome of mutants S200AS203A, S200DS203D, ΔN , ΔC and $\Delta N+C$, with BY4741 and $\Delta knr4$ as the controls. We then tested these mutants with activation *MKK1-P386* by GAL, as well as in the presence of Calcufluor White (CFW) or heat shock. All the transformants grew similarly on the YPD plate and Ura- selective plate as control. On the plate submitted to heat shock, the BY4741 and the truncated Knr4 mutants grew less than

the $\Delta knr4$ and the S200AS203A and S200DS203D mutants. The same result is much more obvious on the plate with CFW and the Gal URA- plate (Figure 33).

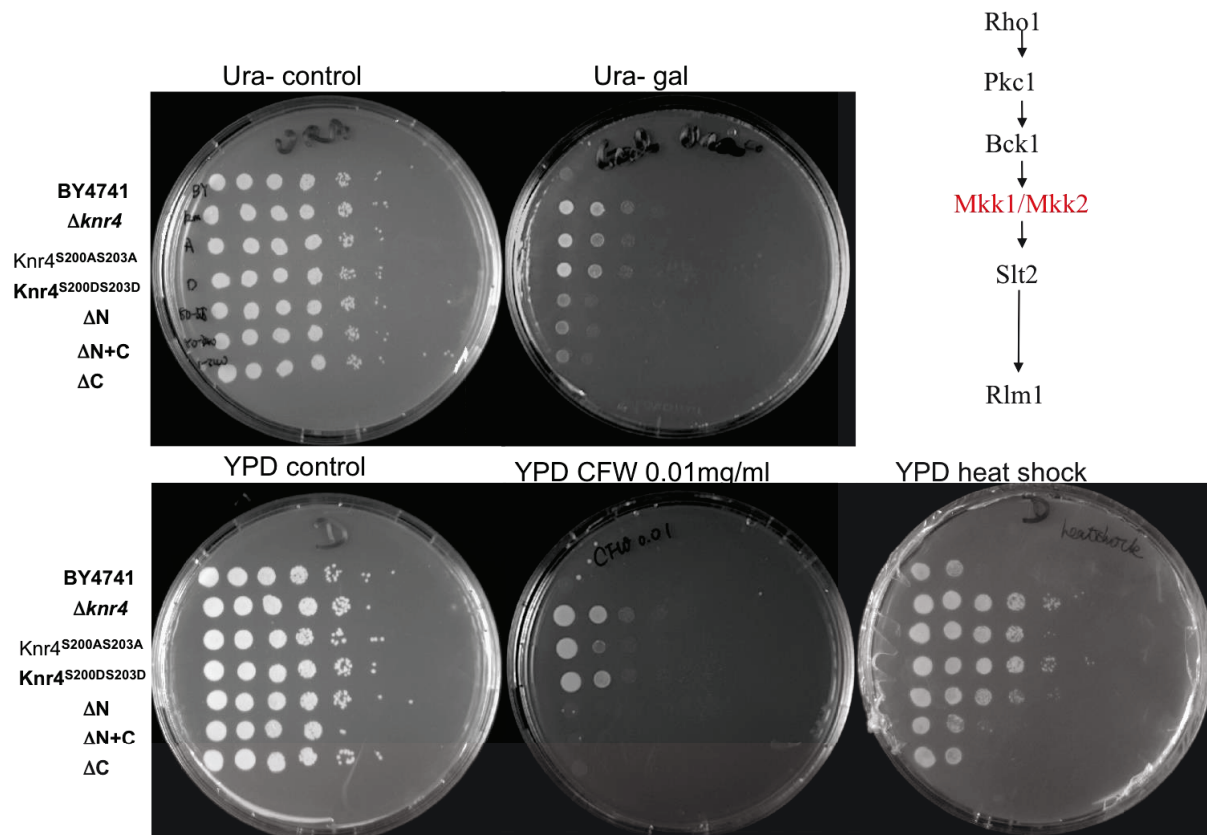


Figure 33 Phenotype of mutants with plasmid p1025 (Mkk1-P386)

Overnight cultures were centrifuged and concentrated or diluted by with sterile the water. The cultures were dropped on the YPD plate and YNB URA- plates as control, and the YPD with GAL and YPD with CFW 0.01mg/ml, and also give the YPD plate submitted to heatshock to these transformants. After 24h, On the YPD control plate, almost all the transformants grow as same. And on the plate from give the heatshock, the BY4741 and the truncated mutants are grew less than the $Knr4\Delta$ and the AA and DD mutants. The same result is obtained on the plate with CFW and the Gal URA- plate.

Remarkably, these CFW sensitivity phenotypes are totally opposite to the phenotype of these same mutants without the plasmid carrying *MKK1-P386* (Figure 34).

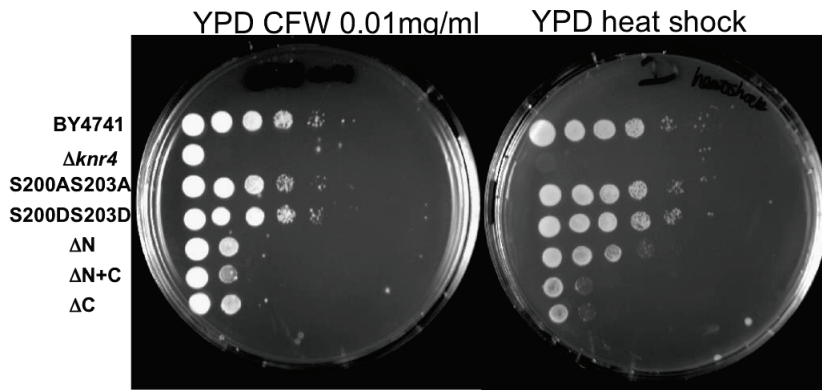


Figure 34 Phynotype of the mutants

For On this phenotype test to CFW, we found that truncated mutants, as *knr4* Δ , are more sensitive than BY4741. We has know Since CFW toxicity is linked to cell wall chitin content, it is believed that n that the *knr4* Δ is sensitive to the CFW because it contains more chitin than BY4741. Because the *knr4* Δ contains more chitin than BY474. Whence, we thought the truncated mutants probably also contains more chitin than the phosphorylation sites mutants. So we tested the amount of the chitin of all these mutants. The results verified our thinking. The truncated mutants are contain more chitin than Phosphorylation sites mutants. (see below).

Our results shows that phosphorylation sites mutants S200AS203A, S200DS203D are able to suppress the growth inhibitory of the Mkk1-P386 like *knr4* Δ mutants, hence towards the hyper activated Mkk1 allele, they behave as the absence of the protein (Figure 33). However, these phosphorylation sites mutants suppress the CFW cells sensitivity just like Knr4 wild type protein (Figure 34) while truncated mutants are very sensitive to CFW and heat shock like *Δknr4*.

Remarkably, among these three truncated mutants, even presence of just the central core of the Knr4 is unable to suppress the Mkk1-P386. Since serine 200 and serine 203 are located in this central core, this further points towards a role of these phosphorylation sites in the suppression of the Mkk1-P386 lethality. As Figure 34 shows, Knr4 is hypersensitive to CFW compared to control strain BY4741, likely because cell walls of *Δknr4* contains much more chitin than wildtype BY4741(Martin et al. 1999). And truncated mutants are similar to the *Δknr4*, while phosphorylation mutants are like wildtype. In order to verify whether these results were indeed correlated to chitin content in cell walls, we measured the chitin of these mutants. The first result shows us that the truncated mutants are similar to the *Δknr4* also and higher than phosphorylation mutants (Figure 35). This result is consistent with the phenotype result of the mutants (without Mkk1-P386 plasmid).

Conclusion:

In our study, we found that serine 200 and serine 203 phosphorylation sites mutants can rescue the *slt2Δknr4Δ* but fail to rescue the *bck1Δknr4Δ*. So the serine 200 and serine 203 residues of Knr4 are important for cell survival in the absence of Bck1. And also our results show us that phosphorylation sites mutants S200AS203A, S200DS203D can suppress the growth inhibitory by the Mkk1-P386 like *Δknr4* mutants. This means that the serine 200 and serine 203 may be involved in the activation of Slr2 by Mkk1. Combining these results suggests that serine 200 and serine 203 residue of Knr4 are involved in the CWI in connection with Mkk1, while activation of Rlm1 by Slr2 may require other phosphorylated residues of Knr4.

µg GNAC residues/mg of purified cell walls

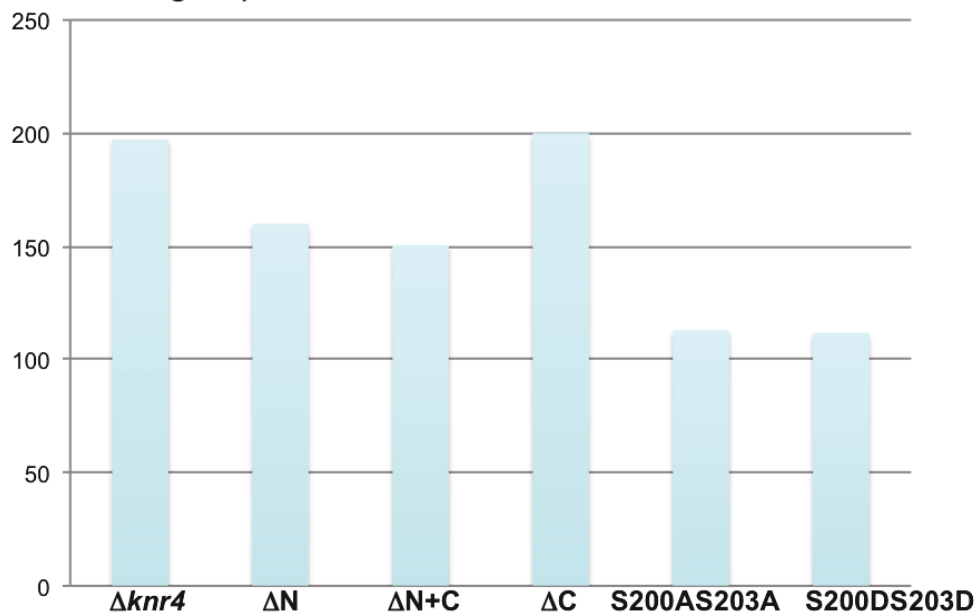


Figure 35 The amount of chitin in the mutants

3.2.4 Influence of Knr4 S200 -S203 and domain deletions on the Calcineurin pathway activation:

The results presented just above (3.2.1) show us that the S200 and S203 phosphorylation sites of Knr4 are important for its interaction with Cna1, which is the catalytic subunit of the PP2B phosphatase complex Calcineurin. So we decided to test the calcineurin activity in the different mutants. The method used for measuring the calcineurin signalling pathway is

described in 2.4.2 of Material and Methods section. . Specifically, we used two vectors: pAL3 and pAL5. Both contain lacZ gene under the dependence of *FKS2* promoter. *FKS2* gene encodes an alternative subunit of the yeast β -(1,3)-glucan synthase. The promoter of this gene responds to various stresses and is controlled by several signal transduction pathways, including Calcineurin pathway. In pAL3, the original copy of the *FKS2* promoter WCE element has been removed, while on the opposite 6 WCEs copies have been integrated by PCR in pAL5 *FKS2* promoter. The plasmid pAL3 is used here as a negative control for Calcineurin induced transcriptional activity. As expected, all mutants and control strains transformed by the control vector pAL3 show no transcriptional activation by calcium in. On the other hand, yeast cells containing pAL5 responded very well to calcium stimulation (see table 8 and the Figure 36). The results show us that without treatment the β -Galactosidase activities measured in all the strains are low and similar. However, this activity is 3-6 times higher in the *knr4* Δ than in BY4741 control strain and the other mutants. After treatment by Calcium which activates the calcineurin pathway and hereby the reporter plasmid, the β -Galactosidase-activities of all the mutants are hundreds times higher than the mutants without treatment. And *knr4* Δ is still significantly higher than the other mutants. Activity measured in the *knr4*^{S200AS203A} is little higher than in *knr4*^{S200DS203D}, which is same as BY4741. All the truncated mutants show similar activity as BY4741, with *knr4* Δ C displaying a β -Galactosidase activity slightly lower than BY4741.

These new data establish that the absence of Knr4 results in hyper activation of the Calcineurin pathway compared to control situation. One of Knr4 cellular functions could thus be the down regulation of this signalling pathway. This could be performed for example by ensuring re-phosphorylation (and hence deactivation) of either Crz1 or Calcineurin itself. The fact that the activity measured in the absence of only the C-terminal domain of Knr4 (= in the Δ C mutant) is lower than in the control BY4741 is also in favour of this hypothesis, since we have shown earlier that removal of Knr4 protein C-terminal domain resulted in a stronger interaction with its protein partners, including Cna1 (Dagkessamanskaia et al., 2010). Remarkably, the fact that the mutant *knr4*^{S200AS203A} shows a transcriptional activity higher than wild type also suggest that serine 200 and serine 203 may be involved in the cross-talking with the calcineurin pathway.

Table 7: β -Galactosidase-Activity of mutants with pAL3 and pAL5

Strains	β -Galactosidase-Activity (nml /min/mg of protein)			
	pAL3		pAL5	
	Without treatment	Calcium	Without treatment	Calcium
BY4741	11,19	38,38	15,43	739,03
<i>knr4</i> Δ	10,53	46,93	125,18	1949,90
<i>knr4</i> ^{S200AS203A}	11,31	36,33	20,90	1399,91
<i>knr4</i> ^{S200DS203D}	14,42	80,16	27,91	780,88
<i>knr4</i> Δ N	12,17	49,93	35,31	1073,07
<i>knr4</i> Δ N+C	10,88	30,85	28,55	872,95
<i>knr4</i> Δ C	9,15	37,81	31,23	571,20

These data are the means from two independent experiments, whose results were similar.

β -Galactosidase-Activity (nml /min/mg of protein)

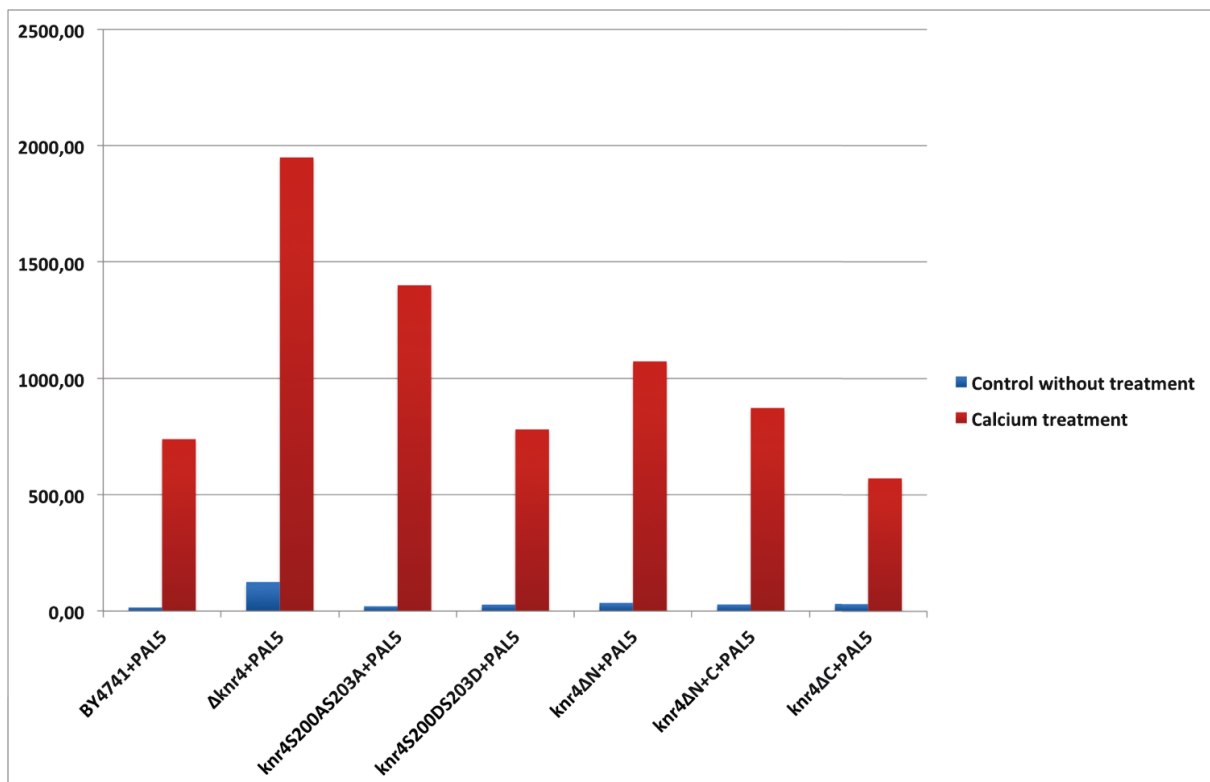


Figure 36 β -Galactosidase-Activity of mutants with pAL5

3.3 The function of Knr4 in the cells sensitivity to the K9 Killer toxin

As explained in the introduction (1.5), K9 killer toxin inhibits the β -(1,3)-glucan synthase of *S. cerevisiae* *in vivo*, on regenerating spheroplasts, and *in vitro* on purified cell membranes. Exposure of whole living *S. cerevisiae* cells to the K9 killer toxin results in the formation of holes in the cell wall, at the tip of growing buds in exponentially growing cells or at the tip of mating projections (or shmoo) in cells exposed to mating pheromones. Stationary phase cells are not sensitive to the killer toxin. The proposed mode of action of K9 killer toxin is the following: the toxin first binds to a complex cell wall receptor structure, progresses through the cell wall, then binds to an unknown protein located on the cell membrane, likely N-glycosylated, and finally inhibits the β -(1,3)-glucan synthase. The initial step, binding to the cell wall receptor structure, is essential to the cytotoxic activity of the toxin, since the toxin does not kill sensitive yeast cells existing as spheroplasts, with no cell walls. Curiously, although spheroplasts survive K9 exposure, their morphology is however affected by the toxin, resulting in perfectly round shaped cells with an increased volume and apparition of huge cellular vacuoles.

Since *KNR4* gene deletion leads to a remarkable resistance to this toxin, we decided to make use of this mutant to attempt to characterize the molecular mechanisms of K9 toxin binding on *S. cerevisiae* cell wall. Mutants defective in *KNR4* gene display reduced β -(1,3)-glucan synthase activity correlated with reduced levels of β -(1,3)-glucan in their cell wall (Hong, Mann, Shaw, et al. 1994a). And, as already reported above, Knr4 protein localizes at the sites of polarized growth such as bud tips and shmoo tips (Martin-Yken et al. 2003; A. Dagkessamanskaia et al. 2010), which are also the places where the toxin forms pores in the cell wall.

In order to better understand the role of Knr4 in the cells sensitivity to K9 killer toxin, we used Atomic Force Microscopy (AFM) to study the nanomechanical properties of the cell wall of *S. cerevisiae* and *knr4Δ* mutant upon exposure to K9 toxin. The results obtained show important modifications of the nanomechanical properties of the cell wall of both control and *knr4Δ* mutant strains after 20 h of K9 treatment, whereas 2 h of treatment caused

modifications only in the case of the wild-type strain. We could therefore hypothesize on the role of Knr4 in the activation of the cell wall remodelling triggered by exposure to K9.

3.3.1 K9 killer toxin treatment modifies the cell wall nanomechanical properties of wild-type cells of *S. cerevisiae*

To explore the K9 killer toxin effects on the Nano mechanical properties of the cell wall of wild type BY4741, yeast cells were first cultivated at 30°C to $OD_{600} = 1$. At this stage, K9 killer toxin was added at a concentration of 0.5 x MIC in order to be able to probe the effects of K9 without killing the cells. AFM analyses were carried out after 2 and 20 hours of incubation, and results were compared to those of control cultures without toxin addition. From the height images presented in Figure 37, average diameters of cells treated with K9 or in native conditions were measured, on at least 5 cells from 2 independent experiments. Our results showed that in the case of the wild-type strain of *S. cerevisiae*, the average diameter of the cells treated with K9 toxin decreased from $4.5 \pm 0.2 \mu\text{m}$ in native conditions to $3.2 \pm 0.6 \mu\text{m}$ after 2 h of exposure to K9 toxin, but did not decrease further upon longer exposure ($3.3 \pm 0.5 \mu\text{m}$ after 20 h). Thus, the morphology of cells is dramatically and rapidly affected by K9 killer toxin. This result was confirmed by a flux cytometry analysis of the cell size among the whole population of K9 treated cultures (see Fig 39).

We then performed nanoindentation experiments in order to determine if this reduced size of the cells was correlated to changes in the nanomechanical properties of the cell wall. Results presented in Figure 37 show that K9 exposure of *S. cerevisiae* wild-type cells causes an increase of the cell wall Young modulus from $512.4 \pm 87.5 \text{ kPa}$ in native conditions, to $853.1 \pm 271.8 \text{ kPa}$ after 2 h of K9 exposure. This increase was even more important after 20 h of exposure of the cells since the Young modulus of the cell wall reached $957.3 \pm 286.0 \text{ kPa}$. Therefore these data clearly show, for the first time, that K9 killer toxin causes an important cell wall remodelling, that might be associated to its known effect to inhibit the β -(1,3)-glucan synthase (Bussey et al. 1979; Shin Kasahara et al. 1994; T. Takasuka et al. 1995). Indeed, in a recent work, it has been reported that treatment of yeast cells with caspofungin, a molecule that also inhibits the synthesis of the β -(1,3)-glucan synthase, results in a decrease in β -(1,3)-glucan compensated by an increase in the chitin content of the cell wall, correlated to a higher cell wall Young modulus (Formosa et al. 2013). The same year, another work showed that caspofungin exposure of *Candida albicans* cells resulted in a major remodelling of this yeast cell wall (El-Kirat-Chatel et al. 2013). Therefore, similarly to the effects of this drug, one can

suggest that the changes of the cell wall Young modulus of cells treated by K9 killer toxin are also due to a remodelling of the yeast cell wall.

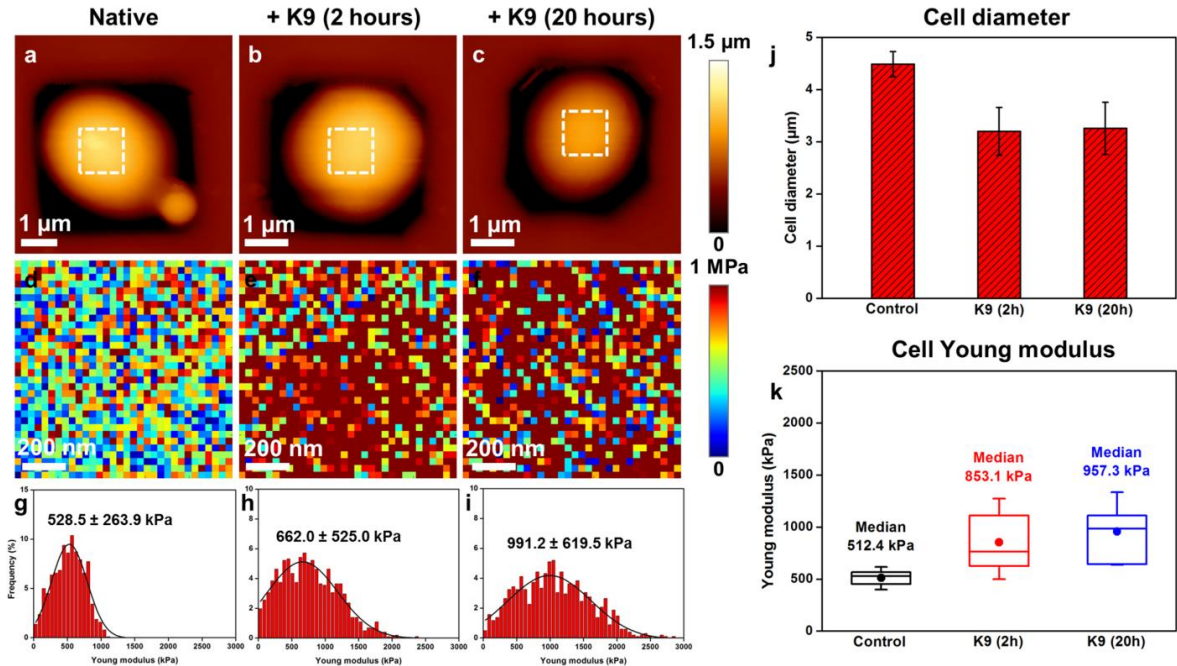


Figure 37: Imaging and nanomechanical properties of *Saccharomyces cerevisiae* strain BY4741 submitted to K9 treatment.

(a) AFM Height image of a single cell immobilized in a PDMS stamp in native condition, (b) treated with K9 killer toxin during 2 hours and (c) treated with K9 killer toxin during 20 hours. (d, e, f) Elasticity maps recorded on areas of $1 \mu\text{m} \times 1 \mu\text{m}$ on top of cells (white squares in a, b and c). (g, h, i) distributions of Young modulus values ($n = 1024$) corresponding to the elasticity maps. (j) Average diameter of cells (statistic on 10 cells coming from 2 independent cultures) and (k) average of Young modulus values (statistics on 10 cells coming from 2 independent cultures).

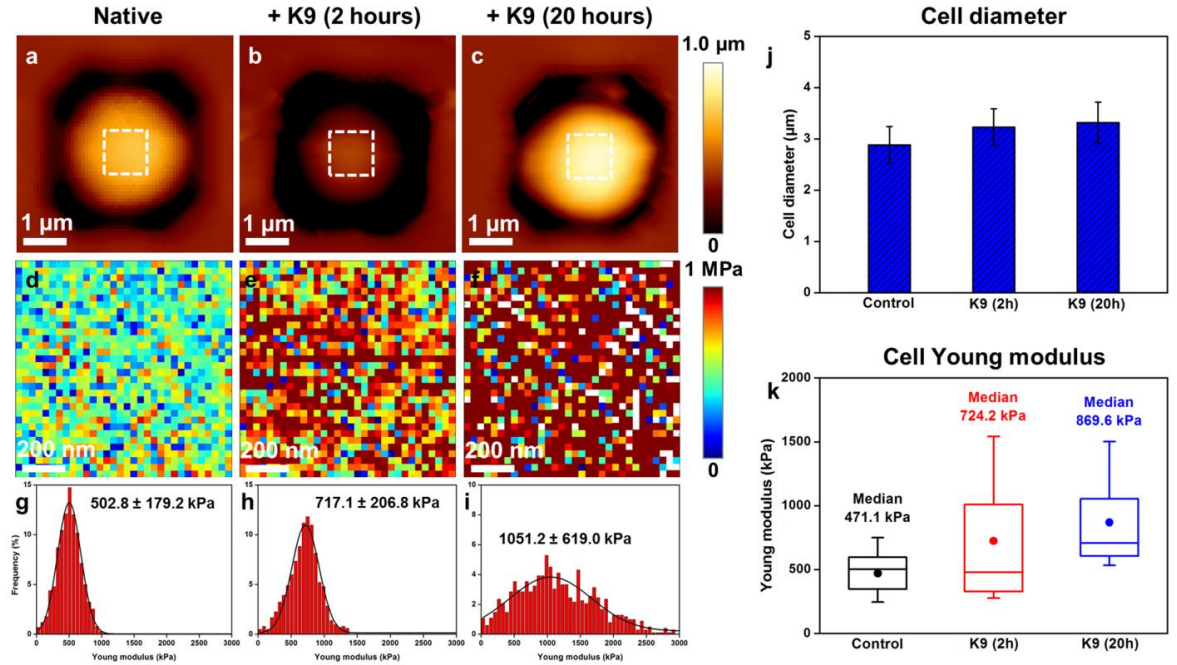


Figure 38: Imaging and nanomechanical properties of *knr4Δ* mutant cells submitted to K9 treatment.

(a) AFM Height image of a single cell immobilized in a PDMS stamp in native condition, (b) treated with K9 killer toxin during 2 hours and (c) treated with K9 killer toxin during 20 hours. (d, e, f) Elasticity maps recorded on areas of $1\ \mu\text{m} \times 1\ \mu\text{m}$ on top of cells (white squares in a, b and c). (g, h, i) distributions of Young modulus values ($n = 1024$) corresponding to the elasticity maps. (j) Average diameter of cells (statistic on 10 cells coming from 2 independent cultures) and (k) average of Young modulus values (statistics on 10 cells coming from 2 independent cultures).

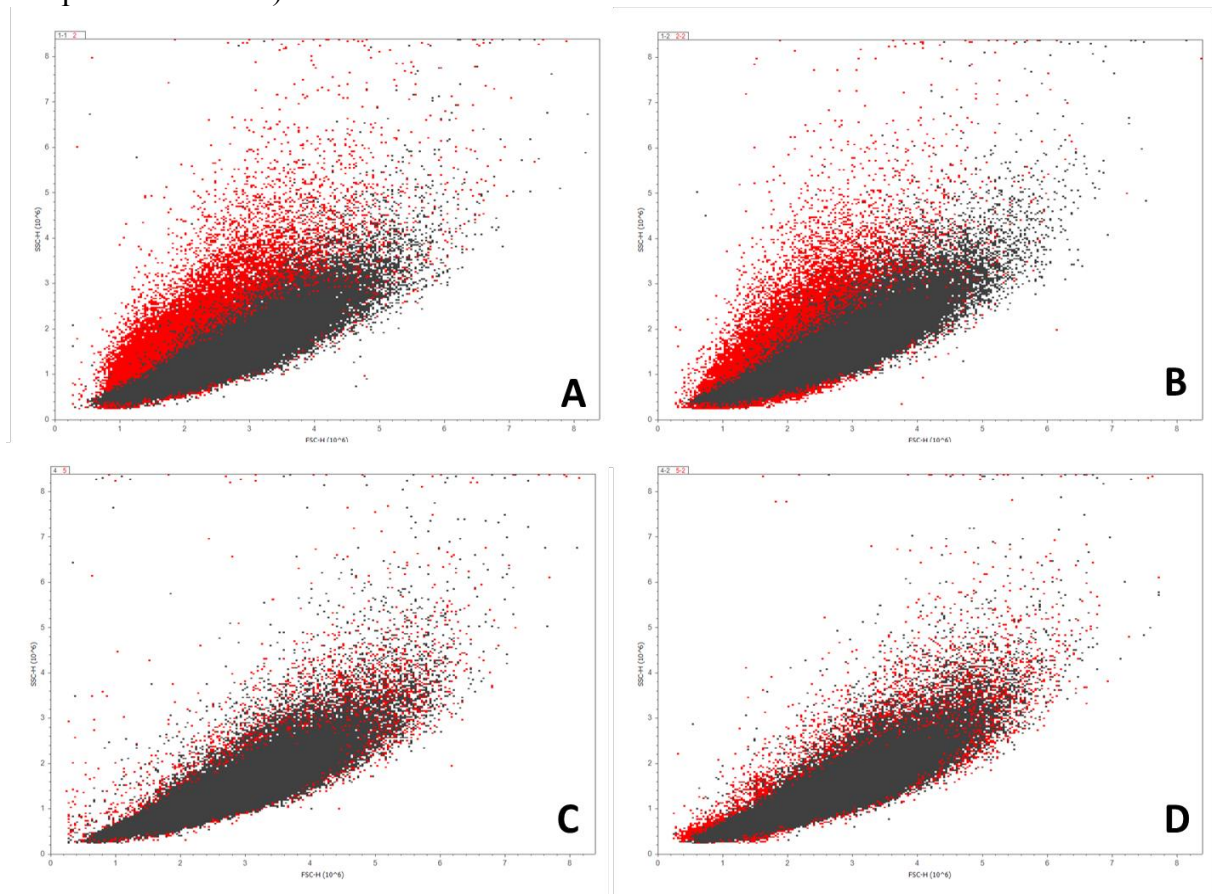


Figure 35: Cytometry Dot-plots.

Red colour spots: cells treated by K9 1/2MIC; Black colour: control cells without K9 treatment.

A and C: obtained after 45min K9 treatment, B and D: obtained after 2 hours K9 treatment.

The X-axe represents the FSC-H (106) whose value reflects the cells size.

The Y-axe SSC-H (106) represents the granularity of the cells, correlated to their complexity.

3.3.2 K9 toxin treatment also affects nanomechanical properties of the toxin resistant *knr4Δ* mutant.

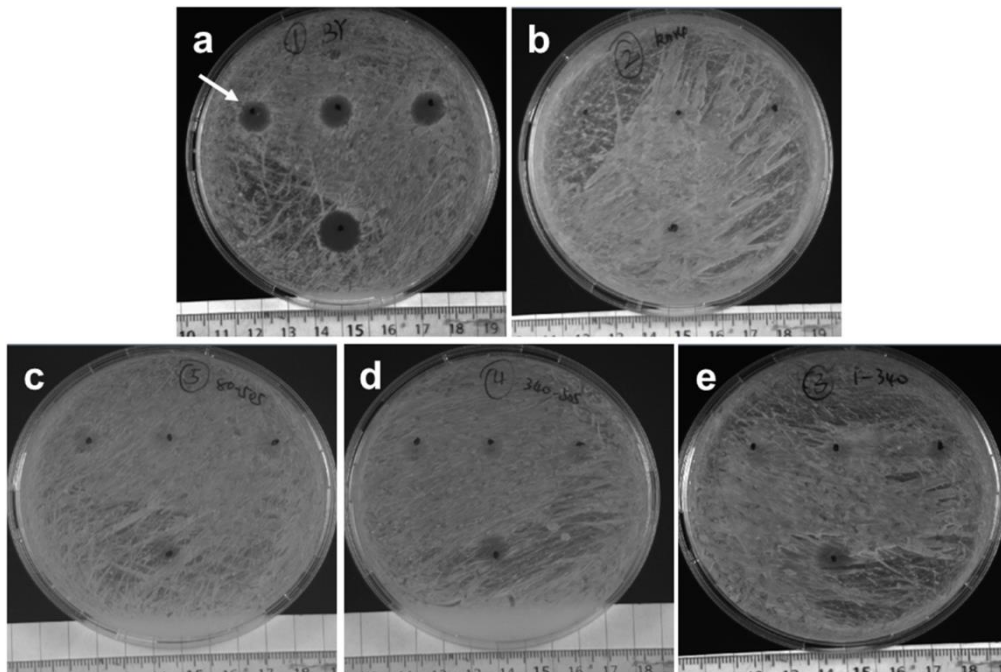
In a further attempt to understand the effects of K9 killer toxin on the cell wall of *S. cerevisiae* cells, we decided to explore the nanomechanical properties of the $\Delta knr4$ mutant. Our initial hypothesis was that $\Delta knr4$ mutant cells, resistant to the K9 killer toxin, would show no or minor modifications of their nanomechanical properties. $\Delta knr4$ cells were cultivated at 30°C to $OD_{600} = 1$, and incubated with K9 killer toxin at $0.5 \times MIC$ for 2 h and 20 h before performing AFM experiments. Our data, presented in Figure 38, point out two distinct results: first, the deletion of *KNR4* gene induces a 30 % reduction of the diameter of the cells in native conditions compared to the wild-type cells ($3.1 \pm 0.5 \mu m$, compared to $4.5 \pm 0.2 \mu m$), which is not further modified by K9 toxin exposure ($3.2 \pm 0.5 \mu m$ after 2h, and $3.4 \pm 0.4 \mu m$ after 20 hours, Figure 38). Therefore, the toxin has no effects on the global morphology of $\Delta knr4$ cells, which is in line with the fact that this strain is resistant to the toxin. Here again, this result was confirmed by flux cytometry analysis of the cell size among the whole culture population (Figure 39). However, when we investigated in more details the effects of the toxin on the cell wall nanomechanical properties, we obtained interesting results. Indeed, in native conditions, cells have a cell wall Young modulus of 471.1 ± 176.8 kPa whereas after 2 h of exposure to K9, the Young modulus is of 724.2 ± 485.9 kPa. The Young modulus therefore appears increased, but these results, recorded on at least 10 cells coming from 2 independent cultures, mostly depict a high variability (standard deviation of 485.9) observed from cell to cell. After 20 h of exposure to K9, the Young modulus of the cell wall is increased to 869.6 ± 346.8 kPa, which is similar to the results obtained on the control strain BY4741 after the same time of incubation. Therefore, K9 seems to also have effects on the cell wall of $\Delta knr4$ mutant, but these effects are visible only after 20 h of treatment. Following our hypothesis that the increase of the Young modulus of the cell wall of *S. cerevisiae* upon K9 treatment is due to an increase in the cell wall chitin content, it seems that this might also happen at 20 hours, in the *knr4Δ* mutant. *KNR4* gene product is known, to repress the expression of genes responsible of chitin synthesis and *knr4Δ* mutant displays a high chitin content already without treatment (Martin et al. 1999). So we expected the cell surface of *knr4Δ* mutant cells to be harder than the one of control cells. But conversely our results show that the *knr4Δ* (471 ± 176.8 kPa in Figure 38) has almost the same cell wall Young modulus as the wild type (512.4 ± 87.5 kPa in Figure 37). This is yet an indication that the Young modulus of the cell wall is not only related to the amount of specific components, but also to

the complex molecular architecture of the yeast cell wall, as shown by our previous analysis (Dague et al. 2010).

3.3.3 Cellular localization of Knr4 protein is required for cell sensitivity to the toxin

The full-length Knr4 protein localizes at the presumptive bud site in G1 and then at the tip of small buds and at the mother-daughter neck during cytokinesis (Martin et al. 1999). Since Knr4 protein localization is reminiscent of the sites of pore formation by the K9 toxin, and since the absence of Knr4 leads to resistance to the toxin, we decided to investigate whether Knr4 localization was required for the cells sensitivity to the K9 killer toxin. Knr4 is a hub protein (Adilia Dagkessamanskaia, Durand, et al. 2010), which interacts genetically and physically with numerous distinct partners (F. Basmaji et al. 2006)(Dagkessamanskaia et al. 2001; Martin-Yken et al. 2003) . Both N-terminal and the C-terminal parts of Knr4 protein are unstructured, and we showed that the N-terminus is required for the protein correct localization at the bud tip (A. Dagkessamanskaia et al. 2010). Three mutant alleles of the gene were created by genomic recombination at the endogenous *KNR4* locus: *knr4 Δ N* (encoding a Knr4 protein without N-terminal domain), *knr4 Δ C* (encoding Knr4 without C-terminal) and *knr4 Δ N+C* (encoding the protein deprived of both N-terminal and the C-terminal). To test the sensitivity of these three mutants to K9 killer toxin, we performed sensitivity halo tests on solid medium, with BY4741 and the Δ *knr4* as respectively positive and negative controls. On the plate where the wild type BY4741 (sensitive to K9) was spread, empty halos formed by growth inhibition can be observed around the places where the K9 killer toxin was deposited (Figure 40A, white arrows). In contrast, no halo at all appeared on the plate on which was spread *knr4 Δ* mutant resistant to K9 toxin (Figure 40B). Remarkably, the mutant *knr4 Δ N* was also found to be much more resistant to the K9 killer toxin than the control strain (Figure 40C). Hence, the N-terminal domain, which is necessary for Knr4 correct localization is also required for the K9 cytotoxic function, in agreement with our hypothesis that localization of the Knr4p is required for the cell sensitivity to the toxin. Unexpectedly, the C-terminal part of the protein may also be involved in this process, although to a lesser extent since *knr4 Δ C* (Figure 40 E) is more sensitive than the *knr4 Δ N* (Figure 40C). This large unstructured C-terminal part of the protein is known to down-regulate Knr4 protein-protein interaction (Dagkessamanskaia, et al. 2010). Hence, it appears that the K9 killer cytotoxic function

requires the correct cellular localization of Knr4 protein and may also involve Knr4



interaction with other protein partners at the sites of intensive cell wall growth.

Figure 40: K9 sensitivity halo test of *Saccharomyces cerevisiae* control strain BY4741 and *knr4* mutants.

(a) B74741, (b) *knr4*Δ, (c) *knr4*ΔN, (d) *knr4*ΔC and (e) *knr4*ΔN+C. K9 killer toxin solution was deposited on the plates as 3 spots of 10μL (upper line) and one spot of 20μL (bottom).

3.3.4 K9 sensitivity of deletion mutants affected in Knr4 protein localization

To furthermore verify our hypothesis without modification of Knr4 protein structure, we decide to make use of the series of genomic deletion mutants in which Knr4 protein localization is lost, that we characterized in the first part of this PhD study: *bem1*Δ, *bem2*Δ, *bni1*Δ, *bud6*Δ, *pcl1*Δ, *pcl2*Δ, *spa2*Δ, *yck1*Δ, *rrd1*Δ, *tpd3*Δ. Then K9 sensitivity halo test were performed on these 10 mutants affected in Knr4 localization, similarly as above. These experiments showed that these mutants are indeed less sensitive to K9 than wild type cells (example on figure 41). These results are in line with the ones obtained with the *knr4*ΔN mutant, and independently support the importance of the correct Knr4 protein localization at

sites of intensive cell wall synthesis during cell cycle, such as the tips of small buds, which corresponds to the places where K9 toxin forms pores in the yeast cell walls (Komiyama et al. 1996).

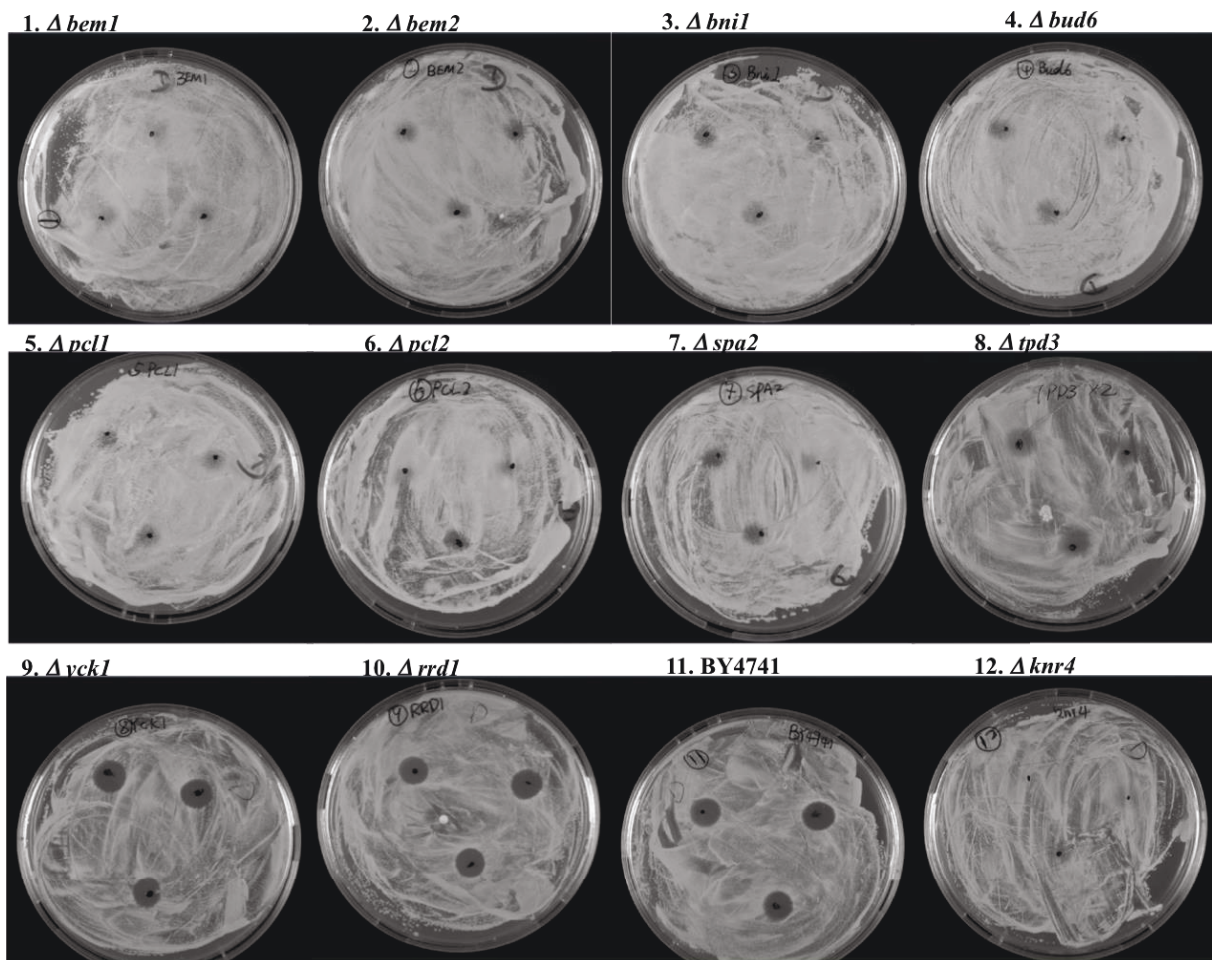


Figure 41: Halo test of K9 sensitivity of 10 the mutants affected in Knr4 cellular localization

Mutants 1 to 8 show decreased sensitivity compared to control 11. BY4741, while mutants 9 and 10 show similar sensitivity as 11. BY4741. $\Delta knr4$ mutant is shown in 12.

3.3.5 Conclusions

Our results constitute the first study of the nanomechanical alterations of the yeast cell surface upon treatment by Killer toxin K9 using Atomic Force Microscopy. Comitini and *al.* (Comitini et al. 2009) used AFM to observe the cell wall surface of yeast treated by another killer toxin, Kpkt produced by *Tetrapisispora phaffii*, but their study was performed on dead and dehydrated yeast cell walls fragments, while we conducted our work on living yeast cells using AFM in liquid conditions. Our results establish a quantitative measurement of the cell

size reduction occurring upon K9 treatment already after 2 hours, as well as of the significant increase in the cell wall Young modulus, representative of a deep cell wall remodelling correlated notably with an increase in chitin content. Moreover, this study allowed us to gain further insights into the mechanism of action of K9 toxin and particularly on its progression from the cell surface towards its cellular target, the β -(1,3) glucan synthase (Miyamoto et al. 2006), which catalytic subunit Fks1 and Fks2 are integral membrane proteins. Even a genome wide search for K9 killer toxin targets did not allow the identification of the putative K9 toxin binding protein (Miyamoto et al. 2011). Thus, we decided to take a different approach and to directly make use of a mutant well known for its resistance to K9 killer toxin, the *knr4 Δ* mutant. We provide here two independent results indicating that the presence of Knr4 protein at sites of intensive cell wall synthesis (*i.e.* bud tips) is necessary for yeast cells to display wild-type sensitivity to the toxin. Hence, Knr4 protein likely participates in the targeting of the toxin towards its target, the β - (1,3) glucan synthase. In order to identify the precise receptor structure responsible for the binding of the K9 toxin at the surface of the cells, further works could involve functionalization of the AFM tip with purified toxin.

4. General conclusion and perspectives

As a hub protein, Knr4 interacts with various partners, which are mainly involved in the cell polarity, signalling pathways, and cell wall synthesis. In our study, we focused on trying to find out the cellular determinants of Knr4 protein localization during the cell cycle and on the function of Knr4 in signalling pathways related to cell wall synthesis.

4.1 Knr4 connections to cell polarity network

It was previously established that Knr4 can interact physically with proteins directly involved in polarized growth including Bem1 and Act1 (Fadi Basmaji et al. 2006). Our study has revealed the involvement of 10 more proteins (**Bem2, Rrd1, Pcl1, Tpd3, Pcl2, Spa2, Yck1, Bem1, Bni1** and **Bud6**) related to polarized growth and morphogenesis, which constitute possible direct or indirect Knr4 partners. **Bem1** is known to establish the cellular polarity that is required for bud and shmoo formation. During mating, Bem1p binds to Ste5 and Ste20, which are central components of the mating pathway, hence one of the Bem1 cellular roles may be to connect the mating signal to the proteins that induce the appropriate changes in the actin cytoskeleton. Taken together, our results and the previous data indicate that Knr4 can interact with the actin cytoskeleton directly or indirectly. Thus, an interesting perspective opened by these results would be to try to look at localization of Knr4 in spheroplasts and with Latrunculin (which induces actin depolarization).

4.2 Function of the Phosphorylation sites of Knr4 in the signalling pathways

Among the 18 *in vivo* phosphorylated sites of Knr4, the phosphorylation sites serine 200 and serine 203 localize in the central core (80-340) of the protein. Our results show that serine 200 and serine 203 phosphorylation sites mutants can rescue the *slt2Δknr4Δ* but fail to rescue the *bck1Δknr4Δ*. In addition, results of our study using Mkk1-P386 hyperactive allele indicate that S200 and S203 may be involved in the activation of Slit2 by Mkk1. On the other hand, the absence of Knr4 results in hyperactivation of the Calcineurin pathway compared to control situation. One of Knr4 cellular function could thus be the down regulation of this signalling pathway. Remarkably, the amino acid replacement of serine 200 and serine 203 in Knr4 also

causes hyperactivation of the Calcineurin pathway compared to the control situation. So we propose that serine 200 and serine 203 in Knr4 may be involved in the cross-talking with the Calcineurin pathway (See Figure 42).

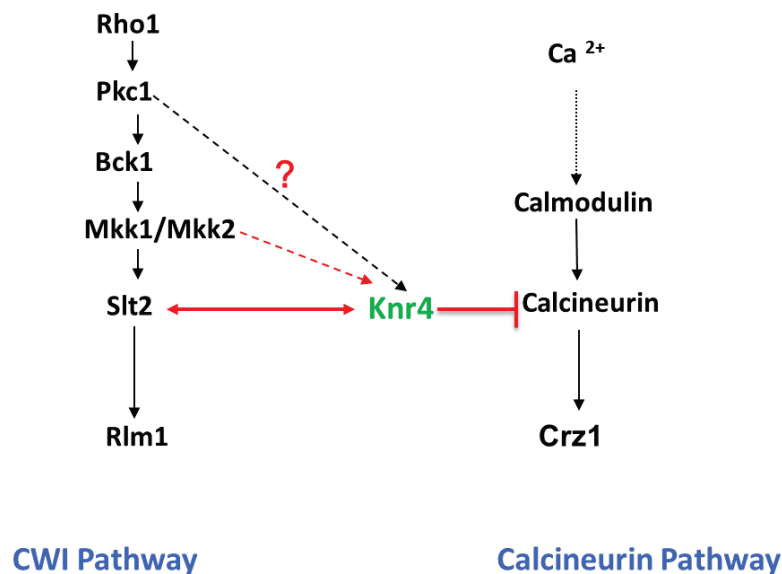


Figure 36: Cross-talk between CWI pathway and Calcineurin pathway

Even though Duand and colleagues found that Knr4 (80-340) can not compensate the synthetic lethality of the *bck1Δknr4Δ* and *slt2Δknr4Δ* double mutants, it is able to rescue *rlm1Δknr4Δ* and *swi4Δknr4Δ*. Martin-Yken and colleagues found that Knr4 is required for the regulation of the kinase activity of Slt2. We thus can speculate that 80-340 domain (or just serine 200 and serine 203) is necessary for Slt2 MAP kinase to activate calcineurin pathway and/or Rlm1 and SBF to maintain cell viability when one of these two signalling pathways is interrupted.

Our results also show that the serine 200 and serine 203 residues of Knr4 are important for cell survival in the absence of Bck1. Thus, it is possible that maybe Pkc1 can interact with Knr4 through serine 200 and serine 203 residues also. This hypothesis would be very interesting to directly test by *in vitro* kinase assay.

4.3. Role of Knr4 in the cells sensitivity to Killer toxin K9

First it was reported that K9 killer toxin inhibits the β -(1,3)-glucan synthase to exert its cytotoxic function to the yeast (Tetsuro Yamamoto et al. 1986; Teturo Yamamoto et al. 1986).

However, the results of global screening did not show that the highly resistant K9 mutants were participating in β -1, 3-glucan synthesis (Miyamoto et al. 2011). K9 is believed to target the Fks1 and Fks2, as the drug caspofungin. And the *FKS1* and *FKS2* deletion mutants show different levels of sensitivity to K9, as they do towards caspofungin. We also found *knr4* mutants are more sensitive to the caspofungin. Remarkably, *fks1* mutant is K9 sensitive but *fks2* shows mild K9 resistant phenotype. Our results show that Knr4 protein likely participates in the targeting of the toxin towards its target, the β - (1,3) glucan synthase, with a major role of Fks2, the second catalytic sub-unit, as one of the main receptors of K9. Further works involving functionalization of the AFM tip with purified toxin may help us to identify the precise receptor structure responsible for the binding of K9 toxin at the cell surface.

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Résumé Long

Rôle de la protéine Knr4 dans la Morphogénèse et la Sensibilité à la toxine killer K9 chez *Saccharomyces cerevisiae*: localisation versus phosphorylation.

La paroi de la levure *Saccharomyces cerevisiae* est une structure très dynamique composée de beta-glucanes, de mannanes et de chitine (polymère de N-acétylglucosamine). Elle peut s'adapter à l'état physiologique et aux changements morphologiques des cellules, ainsi qu'aux contraintes environnementales. Cette remarquable plasticité est assurée par l'intervention de différentes voies de régulation et de signalisation dont la voie CWI (Cell Wall Integrity) et la voie de la Calcineurine ou Protein Phosphatase 2B.

Le gène *KNR4*, dont le nom signifie "*Killer Nine Resistant 4*", a été initialement isolé lors d'un screen de résistance à la toxine K9 Killer de *Hansenula mrakii* (Hong, Mann, Brown, et al., 1994a). Des mutants du gène *KNR4* ont une activité réduite de la β - (1,3) glucane synthase et des niveaux réduits de β - (1,3) - glucanes dans leurs parois (Hong, Mann, Shaw, et al., 1994a).

Chez *S. cerevisiae*, la protéine Knr4 se localise sur les sites de la croissance polarisée tels que les extrémités des bourgeons et des shmoos (Martin-Yken et al 2003;. A. Dagkessamanskaia et al., 2010). Les domaines N- et C-terminal de la

protéine Knr4 sont désordonnés, mais seul le N-terminus est nécessaire pour la localisation de Knr4 à la pointe des bourgeons (A. Dagkessamanskaia et al., 2010). Dans la plupart des fonds génétiques de *S. cerevisiae* le gène *KNR4* n'est pas essentiel pour la croissance dans des conditions de laboratoire standard, mais sa suppression conduit à des défauts de croissance dans de nombreuses conditions de stress telles qu'une température élevée ou la présence de SDS, calcofluor white, congo red, caféine, antifongiques (ex: caspofungine). Cette large gamme de phénotypes corrobore le rôle de la protéine Knr4 dans le contrôle transcriptionnel de l'expression génique (Martin-Yken et al., 2003).

Knr4 est une protéine « HUB » de levure, conservée (Dagkessamanskaia, Durand et al., 2010), et connue pour interagir génétiquement et physiquement avec plusieurs partenaires distincts avec diverses fonctions cellulaires, (Dagkessamanskaia et al 2001 ; F. Basmaji et al., 2006 ; Martin-Yken et al., 2003) (et *Saccharomyces* Genome Database, <http://www.yeastgenome.org>). Grâce à ces interactions, Knr4 influe notamment sur le contrôle transcriptionnel d'un grand nombre de gènes parmi lesquels de nombreux gènes de synthèse de la paroi cellulaire, y compris les trois gènes des chitine synthases présents dans le génome de *S. cerevisiae* (Martin et al., 1999).

En outre, Knr4 participe au contrôle transcriptionnel de la transition G1 / S au cours de la progression du cycle cellulaire (A. Dagkessamanskaia et al., 2010). Enfin, le gène *KNR4* a des homologues dans l'ensemble du génome des champignons, et des données fonctionnelles et génomiques sur les gènes d'homologues de *Candida albicans* (Nett et al., Sd), *Neurospora crassa* (Verdin et al., 2009) et *Schizosaccharomyces pombe* (www.pombase.org) indiquent que leurs produits remplissent des fonctions cellulaires très similaires dans ces organismes.

Ce travail de doctorat s'est essentiellement focalisé sur la fonction de Knr4 dans la synthèse de la paroi cellulaire, la morphogénèse, et les voies de signalisation connexes. Le contenu de ma thèse est principalement divisé en trois parties:

La première partie concerne notre recherche pour trouver des partenaires inconnus de Knr4 et d'étudier les voies cellulaires nécessaires à la localisation de la protéine Knr4. À cette fin, nous avons décidé d'utiliser une série de mutants de délétion interrompus dans les gènes liés à la morphogénèse et l'établissement de la polarité cellulaire. Nous avons sélectionné des gènes candidats de la base de données génomiques de *Saccharomyces cerevisiae* (SGD, Stanford), en utilisant les mots-clés "Morphogénèse" et "la polarité cellulaire". Après sélection, 25 gènes liés à la morphogénèse et polarité cellulaire ont été choisis pour l'analyse de localisation de Knr4. Grâce à l'analyse des résultats, nous avons obtenu 10 mutants intéressants liés à la morphogénèse et la polarité dans laquelle la localisation de Knr4 était affectée: *bem2Δ*, *pcl1Δ*, *pcl2Δ*, *rrd1Δ*, *spa2Δ*, *tpd3Δ*, *bem1Δ*, *bnl1Δ*, *yck1Δ* et *bud6Δ*, ainsi que *pph21Δ* (fonctionnellement lié à Tpd3) et *cna1Δ*, lié à la voie de la calcineurine.

La deuxième partie est constituée d'une analyse mutationnelle de l'effet du rôle des résidus phosphorylés *in vivo* dans la fonction et la localisation de Knr4, ainsi que dans la modulation de l'activité de la calcineurine et la voie CWI. Nous avons trouvé que les mutants de phosphorylation S200S203 ne peuvent pas sauver la viabilité d'un double mutant *bck1Δknr4Δ*, alors qu'ils peuvent sauver *slt2Δknr4Δ*. En outre, des mutants de phosphorylation S200S203 se comportent comme l'absence de Knr4 envers la suppression de la létalité provoquée par un allèle suractivé de la kinase Mkk1. Nous avons constaté de plus que le mutant S200AS203A comme le mutant *knr4Δ* présente une hyperactivation de la voie de la calcineurine par rapport à la souche contrôle. Donc, les serines 200 et 203

peuvent être impliquées dans le contrôle de la voie de la calcineurine et de la voie CWI.

La troisième partie est l'étude de la forte activité cytotoxique de la toxine killer K9 sur les souches de levure sensibles, y compris *Saccharomyces cerevisiae*. Le traitement avec cette toxine conduit à la formation de pores à la surface des cellules, et plus particulièrement aux endroits où la synthèse de la paroi cellulaire est le plus actif, à savoir à l'extrémité des bourgeons de croissance ou des projections de conjugaison. Les cellules de levure traitées par la toxine K9 meurent ensuite en libérant leur cytoplasme et matériaux cellulaires par ces pores. Dans la levure *S. cerevisiae*, la protéine Knr4 se localise sur les sites de la croissance polarisée (bourgeons, shmoos), qui sont aussi les sites où la toxine K9 forme des pores dans la paroi cellulaire. Les mutants défectueux dans le gène *KNR4* sont remarquablement résistants à cette toxine. Dans cette étude, nous avons analysé pour la première fois les effets biophysiques de la toxine K9 sur la paroi cellulaire de la levure en utilisant la microscopie à force atomique (AFM), une technologie de pointe qui permet de mesurer les propriétés nanomécaniques des cellules de levure vivante, et leurs altérations. Nous avons mesuré les effets de la toxine K9 sur les propriétés nanomécaniques de la paroi cellulaire de *S. cerevisiae* de type sauvage et mutants du gène *KNR4*, à court (2 h) et à long terme (20 h). Nos résultats révèlent un important remodelage de la paroi cellulaire se produisant dans les cellules de type sauvage déjà après 2 heures et seulement visible dans le mutant *knr4Δ* après 20 heures de traitement. En outre, nous avons étudié le rôle de la protéine Knr4 de la sensibilité des cellules à la toxine. Nous avons pu montrer que la présence du domaine N-terminal de la protéine Knr4, qui est nécessaire pour sa localisation cellulaire correcte à la pointe des bourgeons au cours du cycle cellulaire, est essentiel pour la sensibilité à la toxine K9. En outre, les mutants de délétion dans lequel la localisation cellulaire Knr4 est perdue (mutants sélectionnés dans la première partie de cette

thèse) présentent une sensibilité réduite à la toxine K9. Dans l'ensemble, ces résultats mettent en lumière l'importance de la bonne localisation de la protéine Knr4 aux sites de croissance intensive de la paroi cellulaire pour la sensibilité des cellules à la toxine killer K9.

1. La localisation de Knr4 dans les mutants de délétion des gènes de morphogénèse

Dans la souche contrôle *S. cerevisiae* BY4741, une fusion C-terminale de Knr4 avec la protéine GFP fluorescente verte (Chalfie et al., 1994) se localise sur le site de bourgeon présomptif en phase G1, puis à la pointe de petits bourgeons et enfin à la jonction mère-fille lors de la cytokinèse (Martin et al., 1999). Le domaine N-terminal de Knr4 est requis pour la localisation cellulaire de la protéine correcte, étant donné que cette localisation est perdue pour la fusion de la GFP de Knr4 (80-505) dépourvue de ce domaine. Ce résultat suggère que Knr4 interagit à travers ce domaine N-term avec au moins une protéine impliquée dans sa localisation. De même, le domaine N-terminal est nécessaire pour la localisation correcte de Knr4 à la pointe des shmoos cours de la différenciation sexuelle. Ces sites de localisation de Knr4 à l'extrémité du bourgeon et la pointe des shmoos sont aussi le lieu de la croissance polarisée. La polarisation initiale de Cdc24 et Cdc42 et la sélection du site de bourgeonnement dépendent fortement de Bem1. Bem1 se co-localise avec Cdc24 et Cdc42 aux sites de croissance, et sa transcription en G1 coïncide avec ces événements de début de polarisation (pour une revue Pruyne & Bretscher, 2000). Nous avons établi précédemment que Knr4 peut interagir physiquement avec les protéines directement impliquées dans la croissance polarisée dont

Bem1 et Act1 (Fadi Basmaji et al., 2006), Dans cette étude, nous avons voulu aller plus loin et avoir une vue globale des protéines partenaires de Knr4 impliqués dans la morphogenèse et la croissance cellulaire polarisée de la levure.

Afin de trouver ces partenaires inconnus et d'examiner les voies cellulaires nécessaires à la localisation de la protéine Knr4, nous avons décidé d'utiliser une série de mutants de délétion interrompus dans les gènes liés à la morphogenèse et établissement de la polarité cellulaire. Nous avons sélectionné des gènes candidats de la base de données du génome de *Saccharomyces cerevisiae* (SGD, Stanford), en utilisant les mots-clés "Morphogénèse" et "Polarité cellulaire". Ce screen a donné 29 gènes différents, auxquels nous avons ajouté *BNII* et *BNRI* codant pour les deux formes de *S. cerevisiae* (nécessaires à la formation des câbles d'actine) et *SPA2* et *BUD6*, éléments du polarisome. A partir de ces 33 gènes différents, après élimination des gènes essentiels (car on ne pouvait pas utiliser des mutants de délétion), ainsi que ceux codant pour des protéines connues pour être localisés ailleurs dans la cellule, 27 gènes ont été conservés pour notre analyse. Sur ces 27, 7 étaient malheureusement absents de la collection Yeast Knock Out (YKO) disponible dans le laboratoire. Les 20 mutants dans les gènes candidats restants ont ensuite été transformés avec un plasmide portant une protéine de fusion Knr4-GFP, et nous avons vérifié si la localisation de Knr4 était conservée, à la fois lors du cycle cellulaire (croissance végétative) et au cours de la différenciation sexuelle. Nous avons utilisé des plasmides qui expriment Knr4 fusionné à la GFP (pHM43) et le plasmide contrôle exprimant la GFP sans Knr4 (pUG35). Après transformation, les différents mutants ont été observés par microscopie à fluorescence.

Après une analyse approfondie des résultats que nous avons obtenus d'abord, nous avons décidé d'observer également Knr4-GFP localisation dans d'autres mutants corrélées aux premiers mutants, comme *cdc55*, *pph21*, *pph22* (liée à

tpd3). Comme nous savons que Knr4 est impliquée dans la voie et calcineurin et la voie CWI, nous avons également vérifié la localisation de Knr4 dans les mutants *cna1Δ* et *slt2Δ*. Enfin, nous avons testé la localisation Knr4 dans les shmoo et bourgeons sur un total de 25 mutants. Par l'observation au microscope à fluorescence nous avons vérifié si la localisation de Knr4 a été conservée pendant la croissance végétative, ainsi que lors de la différenciation sexuelle. Nos résultats montrent que :

- Au cours de la croissance végétative (bourgeon pointe localisation prévu), les produits de gènes *BEM2*, *RRD1*, *PCL1*, *TPD3*, *PCL2*, *SPA2* et *YCK1* sont nécessaires pour la bonne localisation de Knr4.

- Au cours de la différenciation sexuelle (shmoo), dans les mutants supprimés pour les gènes *BEM1*, *BNII*, *TPD3* et *BUD6* la localisation de Knr4 est anormale. Cela suggère que les produits de ces quatre gènes sont nécessaires à la bonne localisation de Knr4.

2. La fonction des sites de phosphorylation et les fragments de la Knr4

Les modifications post-traductionnelles (PTM) des protéines sont des éléments indispensables de la communication cellulaire. Les PTM sont des modifications réversibles, par voie enzymatique des chaînes latérales d'acides aminés spécifiques. Certains types courants de PTM comprennent la phosphorylation et la O-glycosylation sur des résidus Ser (S) et Thr (T), la phosphorylation de Tyr (Y), la méthylation de résidus Arg (R), et l'acétylation et l'ubiquitination de résidus Lys (K). La phosphorylation des protéines décrit la fixation d'un

groupement phosphate sur un résidu spécifique de la protéine, généralement une serine, threonine ou tyrosine ou, plus souvent, de manière transitoire, sur les résidus d'histidine. Les phosphorylations ont la remarquable capacité d'activer et de désactiver la fonction de nombreuses enzymes, modifiant ainsi leur activité, les interactions avec leurs partenaires ou substrat et parfois leur localisation cellulaire. La phosphorylation réversible des protéines est un mécanisme régulateur important qui se produit dans les organismes procaryotes et eucaryotes. Des kinases phosphorylent des protéines et des protéines phosphatases les déphosphorylent. De nombreuses enzymes et récepteurs sont activés "on" ou désactivés "off" par la phosphorylation et la déphosphorylation. La phosphorylation réversible se traduit souvent par un changement de conformation dans la structure d'enzymes et de récepteurs, ce qui provoque leur activation ou désactivation.

Compte tenu de l'implication de Knr4 dans la voie de la PKC1 (une cascade de kinases) et la voie de la calcineurine (un relais de phosphatase), nous avons pensé que la potentielle phosphorylation de Knr4 pourrait être une question pertinente. En conséquence, les études globales de la phosphoprotéome de levure ont révélé 18 dans les résidus *in vivo* phosphorylée en protéines Knr4. Tous ces 18 résidus phosphorylés sont situés sur la partie centrale et C-terminale de Knr4. Le C-terminal, qui a des effets négatifs sur ses interactions avec les partenaires, alors que le noyau central de la Knr4 (AA 80-340) est considéré comme la partie fonctionnelle. Ce noyau central seul est suffisant pour compléter la plupart des phénotypes mutants *knr4*. Il est aussi capable de « sauver » la létalité synthétique des doubles délétions *rlm1Δknr4Δ* et *swi4Δknr4Δ*. Le domaine central de Knr4 est nécessaire pour que la MAP kinase Slt2 active Rlm1, SBF et les autres facteurs de transcription et donc pour maintenir la viabilité des cellules en l'absence de l'un d'eux. Les résidus Serine 200 et Serine 203; situés dans le noyau central de Knr4, ont été proposés pour

être phosphorylée par un autre membre de la voie CWI, Mkk1 qui est la MAP kinase responsable de la phosphorylation *in vivo* de Slt2. Une tentative d'imiter de façon constitutive un état déphosphorylé par la substitution de ces deux résidus sérine par des alanines entraîne la perte de l'interaction physique avec Tys1 (Fadi Basmaji et al., 2006). Nous avons voulu étudier plus avant la fonction de ces deux résidus phosphorylés, nous avons donc utilisé l'acide aspartique (D) pour remplacer les sérines pour imiter un état phosphorylé constitutif de ces deux résidus sérine. Enfin, nous avons eu deux mutants de phosphorylation distincts sur Serine200 et Serine203: Knr4S200AS203A qui imite l'état déphosphorylé, et Knr4S200DS203D qui imite l'état phosphorylé. Ces deux mutants ont ensuite été utilisés comme suit pour étudier la fonction de ces deux résidus. Dans notre étude, nous avons constaté que les mutants de phosphorylation des sérines 200 et 203 peuvent sauver le mutant *slt2Δknr4Δ* mais ne parviennent pas à sauver le *bck1Δknr4Δ*. Ainsi, les résidus sérine 200 et sérine 203 de Knr4 sont importants pour la survie des cellules en l'absence de Bck1. Et aussi nos résultats nous montrent que les mutants des sites de phosphorylation S200AS203A, S200DS203D peuvent supprimer l'inhibition de la croissance par l'allèle hyperactif Mkk1-P386 comme les mutants *Δknr4*. Cela signifie que la sérine 200 et sérine 203 peuvent être impliqués dans l'activation de Slt2 par Mkk1. La combinaison de ces résultats suggère que la sérine 200 et la sérine 203 de Knr4 sont impliquées dans la voie CWI dans le cadre de Mkk1, tandis que l'activation de Rlm1 par Slt2 peut exiger d'autres résidus phosphorylés de Knr4.

3.3 La fonction de Knr4 dans la sensibilité des cellules à la toxine K9

Comme il est expliqué dans l'introduction (1,5), la toxine killer K9 inhibe la β - (1,3) glucane synthase de *S. cerevisiae in vivo*, sur des sphéroplastes en régénération, et *in vitro* sur des membranes de cellules purifiées. Exposer des cellules de *S. cerevisiae* à la toxine K9 résulte dans la formation de trous dans la paroi cellulaire, à la pointe des bourgeons dans les cellules en croissance exponentielle ou à la pointe des shmoos dans les cellules exposées à la phéromone. Les cellules en phase stationnaire ne sont pas sensibles à la toxine killer. Le mode d'action de la toxine killer K9 proposé est le suivant: la toxine se lie d'abord à une structure complexe de récepteur de la paroi cellulaire, progresse à travers la paroi, puis se lie à une protéine inconnue située sur la membrane cellulaire, probablement N-glycosylée, et enfin inhibe la β - (1,3) glucane synthase. La première étape, une liaison à la structure du récepteur de la paroi cellulaire, est essentielle à l'activité cytotoxique de la toxine, puisque la toxine ne tue pas les cellules de levure sensibles à l'état de sphéroplastes, sans parois cellulaires. Curieusement, bien que des sphéroplastes survivent à l'exposition à la K9, leur morphologie est cependant affectée par la toxine, résultant dans des cellules de forme parfaitement ronde avec une augmentation du volume et de l'apparition d'énormes vacuoles cellulaires.

Puisque la délétion du gène *KNR4* conduit à une remarquable résistance à cette toxine, nous avons décidé de faire usage de ce mutant pour tenter de caractériser les mécanismes moléculaires de la toxine K9 sur la paroi cellulaire de *S. cerevisiae*. Des mutants défectueux dans le gène *KNR4* présentent une activité β - (1,3) glucane synthase réduite en corrélation avec des niveaux réduits de β - (1,3) -glucane dans leur paroi cellulaire (Hong, Mann, Shaw, et al., 1994a). Et, comme déjà signalé ci-dessus, la protéine Knr4 se localise sur les sites de la croissance polarisée tels que les extrémités des bourgeons et shmoos (Martin-Yken et al 2003; Dagkessamanskaia et al., 2010), qui sont aussi les endroits où la toxine forme des pores dans la paroi cellulaire.

Afin de mieux comprendre le rôle de Knr4 dans la sensibilité des cellules à la toxine killer K9, nous avons utilisé microscopie à force atomique (AFM) pour étudier les propriétés nano mécaniques de la paroi cellulaire de *S. cerevisiae* et *knr4Δ* mutant lors de l'exposition à la toxine K9. Les résultats obtenus montrent des modifications importantes des propriétés nanomécaniques de la paroi cellulaire du contrôle et de souches mutantes *knr4Δ* après 20 h de traitement K9, alors que 2 h de traitement a provoqué des modifications uniquement dans le cas de la souche de type sauvage. Nos résultats constituent la première étude des altérations nanomécaniques de la surface de la cellule de levure lors d'un traitement par la toxine tueuse K9 utilisant la microscopie à force atomique. Comitini et al. (Comitini et al., 2009) ont utilisé l' AFM pour observer la surface de la paroi cellulaire de la levure traitée par une autre toxine tueuse, Kpkt produite par *Tetrapisispora phaffii*, mais leur étude a été réalisée sur des fragments de paroi de cellules de levure mortes et déshydratées, alors que nous avons effectué notre travail AFM sur des cellules de levure vivantes dans des conditions liquides. Nos résultats établissent une mesure quantitative de la réduction de la taille des cellules se produisant lors d'un traitement K9 déjà au bout de 2 heures, ainsi que l'augmentation importante du module de Young de la paroi cellulaire, représentatif d'un profond remodelage de la paroi cellulaire, corrélé avec une augmentation de sa teneur de la chitine. En outre, cette étude a permis d'acquérir de nouvelles informations sur le mécanisme d'action de la toxine K9 et en particulier sur sa progression à partir de la surface cellulaire vers sa cible cellulaire, la β - (1,3) glucane synthase (Miyamoto et al., 2006), qui dont les sous-unité catalytique et Fks1 Fks2 sont des protéines membranaires intégrales. Même un crible génomique de recherche de cibles de la toxine killer K9 n'a pas permis l'identification de la protéine de liaison putative de la toxine K9 (Miyamoto et al., 2011). Ainsi, nous avons décidé de prendre une approche différente et d'utiliser un mutant bien connu pour sa résistance à K9 toxine tueuse, le mutant *knr4Δ*. Nous fournissons ici deux résultats indépendants

indiquant que la présence de la protéine Knr4 sur les sites de la synthèse de la paroi cellulaire intensive est nécessaire pour que les cellules de levure affichent une sensibilité à la toxine de type sauvage. Par conséquent, la protéine Knr4 participe vraisemblablement au ciblage de la toxine vers la β - (1,3) glucane synthase. Afin d'identifier la structure précise des récepteurs responsables de la liaison de la toxine K9 à la surface des cellules, d'autres travaux pourraient impliquer la fonctionnalisation de la pointe de l'AFM avec la toxine K9 purifiée.

Mots-clés: la levure *Saccharomyces cerevisiae*, Knr4, La voie CWI, Localisation, Phosphorylation, K9, AFM.