Trehalose accumulation and the Hxt5-Tps1 complex in *Saccharomyces cerevisiae*

Role in cell cycle elongation and glucose metabolism

Trehalose accumulatie en het Hxt5-Tps1 complex in *Saccharomyces cerevisiae*

Rol in celcyclus verlenging en glucose metabolisme

(met een samenvatting in het Nederlands)

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Voor mijn ouders,
voor David

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List of Abbreviations

AMP	Adenosine mono-phosphate
ATP	Adenosine tri-phosphate
cAMP	Cyclic AMP
CDC	Cell Division Cycle
CDK	Cyclin-dependent kinase
CKI	Cdk inhibiting protein
DAG	Diacylglycerol
ER	Endoplasmic reticulum
GFP	Green fluorescent protein
GLUT	Glucose transporter
HA	Hemagglutinin
HAP	Hap2/3/4/5 protein
Hxk	Hexokinase
Hxt	Hexose transporter
MAP	Mitogen-activated protein
MAPK	MAP kinase
MAPKK	MAPK kinase
MAPKKK	MAPKK kinase
MBF	MCB binding factor
MCB	Mlu1 cell cycle box
PDS	Post diauxic shift
PEST	region enriched in proline (P), glutamine (E), serine (S) and threonine (T) residues
PKC	Protein kinase C
PL	Phospholipid
PLC	Phospholipase C
SBF	SCB binding factor
SCB	Swi4,6-dependent cell cycle box
STRE	Stress-responsive element
TOR	Target of Rapamycin
Ub	Ubiquitin

Chapter 1

General Introduction

Introduction

The baker's or budding yeast *Saccharomyces cerevisiae* is able to survive under many different environmental conditions. Nutrient availability is one of the environmental factors to which the cells have to adapt to. The cells are able to respond to changes in their environment and under influence of the extracellular conditions, they alter their cell size and cell cycle length (1-3). When conditions are not favourable enough to enter the next cell cycle, haploid cells enter a quiescent state and diploid cells form four haploid spores, which can survive long under extreme conditions (4, 5).

The present chapter describes briefly the genes, proteins and processes studied in this thesis and is followed by the outline of this thesis. The cell cycle together with an involved signal transduction pathway, the methods for synchronization and cultivation, and glucose transport together with reserve carbohydrate metabolism are described in more detail in Chapter 2.

The cell cycle

The cell cycle can be divided into four phases. During the first Gap (G1) phase, cells grow and prepare themselves for a new cell cycle. The decision to enter the cell cycle or to enter a quiescent state is taken at the end of the G1 phase and is located at a point called 'Start' (6). Exposure of haploid cells to mating factor of the opposite type can also arrest cells before start (7). Once cells have passed Start, they have committed themselves to complete the cell cycle before they can enter the quiescent state. After Start, cells progress to the DNA Synthesis (S) phase. In the S phase, DNA is replicated once, and this is checked during the second Gap (G2) phase. In the G2 phase, the cells also monitor their morphology to prevent binucleated cells or cell lysis. The last phase is Mitosis (M phase) and in this phase the chromatids are separated and two new cells are formed.

In contrast to most cells, *S. cerevisiae* cells divide asymmetrical with a large mother cell and a small daughter cell (8). In wild type cells, entry into S phase is characterized by the appearance of the bud, which grows during the S, G2 and M phase until it is cleaved from the mother cell at the end of the M

phase. After mitosis, the mother cells enter the next cell cycle almost immediately, while the daughter cells have to grow in size first.

Cyclins and cyclin-dependent kinases

Progression through the cell cycle is driven by the activity of cyclins complexed to cyclin-dependent kinases (Cdk's). In *S. cerevisiae*, Cdc28p is the only Cdk required for cell cycle progression (9). The cyclins that bind to Cdc28p define the different phases of the cell cycle. During the G1 phase, the G1 cyclins Cln1p, Cln2p and Cln3p activate Cdc28p (10). Clb5p and Clb6p are complexed to Cdc28 during S phase (11). Clb1p, Clb2p, Clb3p and Clb4p are the mitotic cyclins and are required for progression to and through M phase (12).

Regulation of G1 phase progression

CLN3 is the first cyclin expressed and transcribed during the G1 phase, and Cln3p binds to and activates Cdc28p (13, 14). Via activation of the transcription factor SBF (SCB (Swi4,6-dependent cell cycle box) binding factor), Cln3-Cdc28 activates transcription of *CLN1* and *CLN2* (13-15). The SBF transcription factor complex consists of the DNA binding protein Swi4 and the regulatory protein Swi6 (16). Cln1-Cdc28 and Cln2-Cdc28 induce transition to S phase by stimulation of DNA replication and by positively influencing bud formation (17, 18). During the G1 phase, the Cdk inhibitor (CKI) Sic1 inhibits Clb5p and Clb6p activity (19). Sic1p is phosphorylated by Cln1-Cdc28 and Cln2-Cdc28 and subsequently ubiquitinated and degraded (19, 20). After degradation of Sic1p, Clb5-Cdc28 and Clb6-Cdc28 initiate DNA replication.

Pkc1-Slt2 cell wall integrity pathway

One of the yeast signal transduction pathways is the mitogen activated protein kinase (MAPK) Pkc1-Slt2 cell wall integrity pathway. This pathway is involved in maintenance of the cell wall and adaptation of the cell wall to changes in environmental en intracellular conditions. Cdc28p induces the

activity of the Pkc1-Slt2 cell wall integrity pathway during the G1 phase. The pathway consists of the upstream kinase Pkc1p, which is activated either via receptors or via diacylglycerol (DAG), which is released under influence of Cdc28 (21-26). Pkc1p activates Bck1p, which in turn activates Mkk1p and Mkk2p. These two redundant kinases activate the most downstream kinase, Slt2p (27-30).

Active Slt2p can phosphorylate Swi6p and this modification induces the export of Swi6 out of the nucleus into the cytoplasm. Swi6p is localized to the nucleus during the G1 phase, but is cytoplasmatic during the other phases of the cell cycle (31, 32). The transcription of cell wall genes requires only the presence of *SWI4* (33). The controlled localization of Swi6p might therefore enable a cell to control cell cycle progression in both DNA replication and cell wall modulation.

Cell synchronization

Cell cycle studies mostly require that cells are synchronized at a specific phase of the cell cycle and this can be obtained in several ways. The cells can be arrested in a phase until all cells have reached that phase or the cells in a specific phase are isolated from the rest of the population. The first method uses chemicals, temperature-sensitive mutants, deprivation of factors essential for growth, or synthetic mating factor (34-37). These synchronization methods all interfere with normal cell cycle progression. The second method uses the differences in size of *S. cerevisiae* to synchronize cells by elutriation (37, 38). A combination of centrifugal force and medium flow enables this method to specifically collect daughter cells in early G1 phase and this synchronization is used in this study. It is also possible to collect cells of all phases of the cell cycle by elutriation by increase of the medium flow and collection of fractions of the outflow.

Nutrient availability and G1 phase length

Growth of synchronous early G1 phase cells in fed-batch cultures revealed a correlation between the length of the G1 phase and the amount of

nutrients in the feed. The G1 phase was slowly elongated with decreasing galactose fluxes until a switch point was reached. When the galactose flux was set below that switch point, the G1 phase was rapidly elongated (3, 39). The increase in G1 phase elongation was accompanied by the accumulation of the reserve carbohydrates trehalose and glycogen (39).

Reserve carbohydrates trehalose and glycogen

Accumulation of reserve carbohydrates is important for cell viability. The complex consisting of Tps1p, Tps2p, Tps3p and Tsl1p is responsible for the accumulation of trehalose (40-45). Glycogen is accumulated by action of Glg1p, Glg2p, Gsy1p, Gsy2p ad Glc3p (46-48). Cells are no longer able to accumulate trehalose after deletion of *TPS1* and no longer able to accumulate glycogen by deletion of *GSY1* together with *GSY2*.

The function of Tps1p

Cells deleted for *TPS1* are not only unable to accumulate trehalose; they are also unable to grow on glucose, fructose or mannose. Addition of glucose to *tps1*Δ cells leads to hyperaccumulation of early glycolysis intermediates together with decreased levels of ATP and inorganic phosphate (49, 50). It was suggested that Tps1p functions as a regulator for import of glucose into the glycolysis or into the cell by regulation of hexose transporter (Hxt) proteins (40, 44, 51).

Hxt proteins

Glucose is transported into the cell by a family of hexose transporter (Hxt) proteins. Of this family, *HXT1-4* and *HXT6/7* are the major hexose transporters and their expression is regulated by the extracellular glucose concentration (52-59). *HXT5* is also capable of transporting glucose but its expression is regulated by the growth rate of the cells (60, 61).

Outline of this thesis

The aim of this thesis was to gain insight into the regulation of G1 phase progression, into the regulation of trehalose accumulation and into the metabolism of Hxt5p, which appeared to be involved in trehalose accumulation.

In Chapter 2, the main genes, proteins and signal transduction pathways, which are involved in the processes described in this thesis, are reviewed.

The role and requirement of the kinase *SLT2* for cell cycle progression was investigated and this study is described in Chapter 3. To localize the timing of Slt2p activity in the cell cycle, the protein expression pattern of several key players and the activity of the Slt2 during short and elongated G1 phases are described. The results indicate that the protein expression patterns of Cln1p are dependent on the carbon source and the length of the G1 phase, while Cln3p is independent on those variables. The activity of Slt2p was linked to the length of G1 phase and deletion of *SLT2* resulted in severe cell cycle elongation.

In Chapter 4, the interplay between hexose transporters, trehalose accumulation and *TPS1* expression is examined. Accumulation of trehalose correlated most with the expression of *HXT5*. The accumulation of trehalose was not dependent on one hexose transporter in particular. Although localization of Hxt5-HA by immunofluorescence microscopy indicates plasma membrane localization, electron microscopy on cryosections revealed that Hxt5-HA is predominantly localized at the endoplasmic reticulum. The localization of Hxt5p in the endoplasmic reticulum might indicate a role in gluconeogenesis as this is the place of the final step in gluconeogenesis in mammalian cells and a glucose transporter is also present in the protein complex involved in gluconeogenesis in the ER of mammalian cells. The interaction of Hxt5p and Tps1p suggested a role for Hxt5p in the accumulation of trehalose under conditions of gluconeogenesis or restriction of glucose influx into the cell by Tps1p activity.

The role of the complex with Hxt5p and Tps1p was investigated in Chapter 5. Deletion of *TPS1* prevented cells from expression of the major hexose transporters on glucose medium, and *HXT5* expression was only detected on glucose medium when *HXT5* mRNA was already present in the initial culture. The decrease in expression of the housekeeping genes suggests that *tps1*Δ cells are unable to express mRNA at all. The immediate decrease in trehalose levels after glucose addition to starved cells indicated a vectorial shuttling of glucose from outside the cell to either glycolysis or carbohydrate accumulation.

The localization and the degradation process of Hxt5p was examined and this study is described in Chapter 6. Ubiquitination of Hxt5p was not detected although the presence of a PEST-sequence and phosphorylation on at least one serine residue suggested otherwise. The coexistence of the Hxt5-Tps1 interaction and the Hxt5 phosphorylation might however indicate a role for the serine phosphorylation in the regulation of Hxt5p activity. In addition, Hxt5p was only phosphorylated during a short time after induction of the growth rate, which was caused by addition of glucose, while the majority of Hxt5p degradation occurred later. Hxt5 was degraded in the vacuole after internalization via endocytosis as observed by electron microscopy.

In Chapter 7, the studies in this thesis are placed in a broader perspective and the results described are discussed.

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Chapter 2

Nutrient driven cell cycle progression in *Saccharomyces cerevisiae*

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Abstract

Cell cycle progression in *Saccharomyces cerevisiae* is, like in all cells, a tightly controlled process. Work in our group has focused on how the progression through the G1 phase is regulated. Accumulation of the reserve carbohydrates trehalose and glycogen are shown to be important for elongation of the G1 phase when the cells are grown at low galactose concentrations. The glucose transporter Hxt5p is suggested to play a role in accumulation of trehalose and this is probably under control of the cAMP-PKA pathway. This pathway positively influences early G1 phase progression, while a model is set up that suggests that the *PKC1-SLT2* pathway is important for late G1 to early S phase progression.

Introduction

Knowledge on the molecular mechanisms that govern the progression through the cell cycle has increased considerably during the last decades, mainly because this knowledge is fundamental for understanding the cause and subsequent treatment of many diseases, including cancer. Cell cycle research in yeast has played a prominent role in these developments, due similarity in regulation of cell cycle progression between yeast and higher eukaryotes, and the advantages of yeast as model organisms because of the readily applicable molecular biological approaches and defined and easy culture conditions. Cell cycle regulation is usually determined by external factors, and the main difference between yeast and higher eukaryotes concerns the different properties of these external factors. In yeast, cell cycle progression is largely determined by the availability of nutrients and to a lesser extent by specific signal molecules such as pheromones. In mammalian cells, cell cycle progression is determined by growth factors, extracellular matrix factors and cell-attached signal molecules, and to a lesser extent by nutrients. However, the intracellular signal transduction cascades that transmit the external signal from the plasma membrane to the nucleus are largely comparable. This homology in signal transduction systems and the homology in the molecular systems that govern cell cycle progression make the yeast cell

still an attractive organism to unravel the molecular systems that regulate cell cycle progression by nutrients. In this review we will describe briefly the current knowledge on cell cycle regulation in *Saccharomyces cerevisiae* and in more detail the mechanisms governing nutrient-determined cell cycle progression.

Cyclins and Cyclin-dependent kinases.

The cell division cycle or cell cycle can be divided in four successive phases: the G1 (or Gap1) phase, the S (or Synthesis) phase, the G2 (or Gap2) phase and the M (or Mitotic) phase (figure 1). Unlike other eukaryotic cells, the division of *S. cerevisiae* is asymmetric, with a relative large mother and a small daughter cell. After division, the mother cell will be able to start a new cell cycle (almost) immediately, while the daughter cell has to grow in size before it can enter the S phase. Environmental conditions determine the (specific) cell size at which cells will leave the G1 phase and enter the S phase. This size threshold increases in proportion to cell ploidy and decreases when more nutrients are available. When conditions are not favourable, haploid cells will enter a quiescent state, while diploid cells will undergo meiosis and form four haploid spores that can survive long under extreme conditions. If conditions are favourable, cells will start a new cell cycle. The characteristic sign that *S. cerevisiae* cells have entered S phase is the appearance of a bud (1). This bud will continue to grow during the S and G2 phase, until it is separated from the mother at the end of mitosis. The decision whether to enter a new cell cycle or to enter the quiescent state is taken at the end of the G1 phase, at a point called 'Start' (2). After cells have passed Start, cells initiate DNA replication, spindle pole body duplication and bud growth. These events are critical for further cell cycle events, namely mitosis and cytokinesis (reviewed in (3)). Prior to the execution of Start, nutrient limitation or mating pheromones can arrest cells in G1 (4).

Cell cycle progression is dependent upon the activity of molecular cell cycle mediators, the cyclin dependent kinases (Cdk's). One single Cdk, Cdc28p, is sufficient and required for regulation of cell cycle progression in *S.*

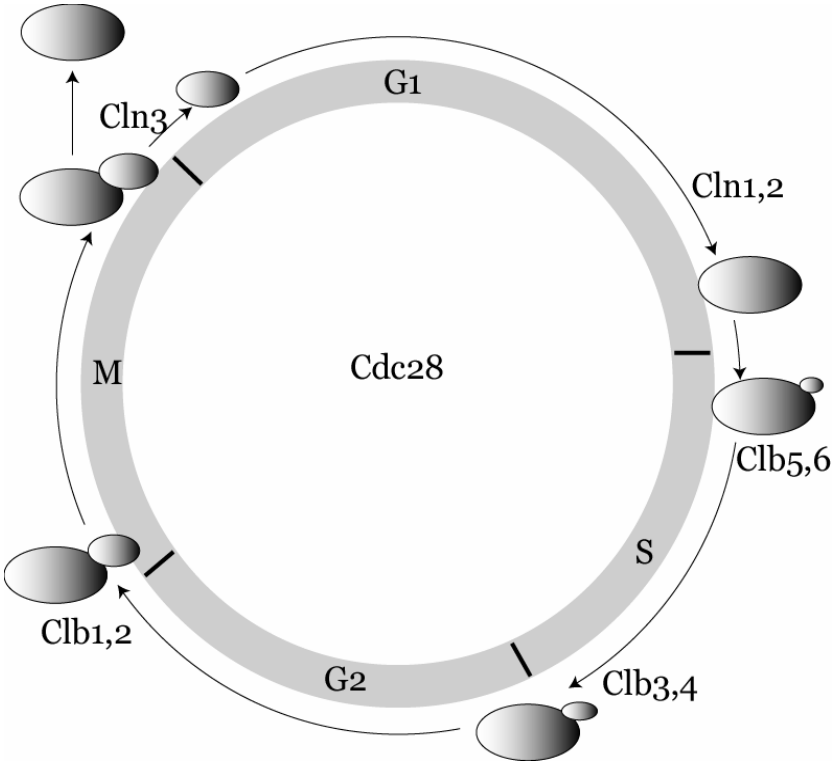

Figure 1: Cell cycle of *Saccharomyces cerevisiae*. Daughter cells start the cycle as small cells and grow during G1 phase. *CLN3* is constantly expressed and during G1, the Cln3-Cdc28 complex induces transcription of *CLN1* and *CLN2*. Cln1,2-Cdc28 complexes induce passing of Start and entry into S phase which is characterized by appearance of a bud. During S phase, *CLB5* and *CLB6* are expressed. Clb5,6-Cdc28 complexes induce replication of DNA and are required for S phase progression, while Clb1-4 are important for progression through G2 and M phase.

cerevisiae. Regulation of Cdc28p activity is dependent upon the interaction with cyclins. Cyclins are a family of proteins that share limited homology at the primary sequence level in a region called the 'cyclin box'. This cyclin box encodes a structural motif that is required for interaction with and activation of the Cdk partner. After binding to the cyclins, Cdc28p activity is regulated further by a combination of activating and inhibiting phosphorylations and the possible interaction with Cdk inhibiting proteins (CKIs). At different stages of

the cell cycle, different cyclins have to interact with and activate Cdc28. The G1 cyclins, Cln1p, Cln2p and Cln3p, activate Cdc28p during G1 phase. Clb5p and Clb6p are required for S phase progression and Clb1 through 4 are the mitotic cyclins, and are necessary for progression through the M phase.

In addition to Cdc28, *S. cerevisiae*, contains four other Cdk's: Pho85p, Kin28p, Srb10p and Ctk1p. These Cdk's are not essential for cell cycle progression, but contribute to it by regulating gene expression and metabolism. Pho85p is the only other kinase that associates with multiple cyclins, the other three Cdk's interact with only one cyclin and regulate gene expression by close links to the transcriptional machinery (reviewed in (5)).

Cell synchronization and fed-batch culturing

In order to be able to study progression of cells through the cell cycle, in most instances the cells have to be synchronized. Synchronized populations of *S. cerevisiae* can be obtained by several methods. These methods can be divided into two categories: either the cells are arrested in a certain phase until the entire population resides at the same point of the cell cycle, or cells in the same phase are isolated from the remaining population.

The addition of α-factor leads to cell cycle arrest in G1 and is a common method to synchronize *S. cerevisiae*. However, besides arresting the cells at G1, the exposure to α-factor leads also to changes in the regulation of mating behaviour and probably also influences other regulatory pathways (6). Another way of synchronizing yeast cells is by using a temperature sensitive *cdc15* mutant. Cdc15p is a kinase participating in a MAP kinase pathway, and has been demonstrated to be required for Clb2/Cdc28 kinase inactivation after mitosis (7). By shifting the culture to the restrictive temperature, the Cdc15 protein becomes inactive, which leads to cell cycle arrest in late mitosis. A disadvantage using this method is that the applied temperature shift to 37°C, required for the inactivation of Cdc15, may also lead to a heat-shock response. Other methods of cell synchronization include deprivation of essential factors

required for growth (8), or by the exposure to certain specific inhibitors (9), which block events required for progression through specific cell cycle phases.

Since the newly formed daughter cells are usually small, as compared to the mother cells, cells can be synchronized using an elutriator.

Controlled progression through the cell cycle of synchronized cells can be achieved by fed-batch culturing of the cells (10). For example, synchronous cells obtained by elutriation, were incubated for a short period in minimal medium without a carbon source. Subsequently, a low amount of galactose was added to the culture with a constant rate. Under these conditions the residual galactose concentration remained virtually constant and consequently also the galactose consumption rate is constant. Using this method, the length of the G1 phase is determined by the galactose concentration in the feed, as demonstrated by the expression of the S-phase gene *H2A* and the budding percentage of the cells (figure 2.).

The precise control of the length of the G1-phase using this fed-batch system gives the opportunity to study factors influencing cell cycle progression. In figure 3 is shown how the expression of cell cycle specific transcripts of *CLN1, CLN2* and *CLN3* varies when G1-phase is elongated by limiting the amount of available galactose. In the upper part is the progression through the

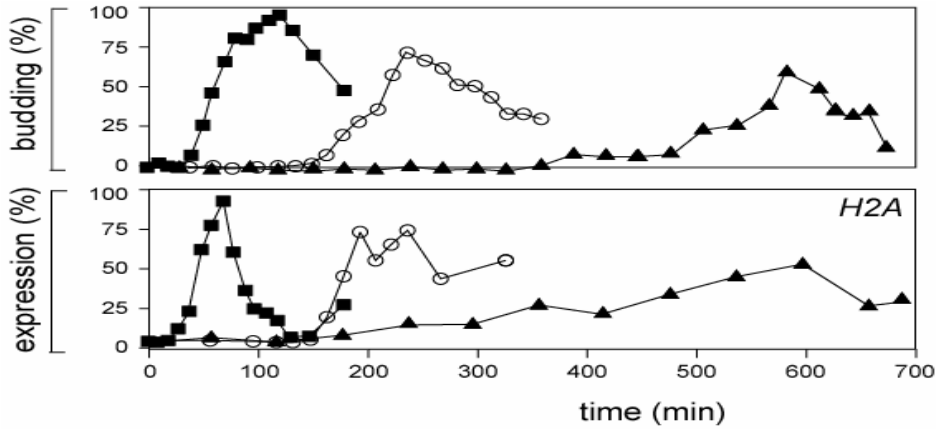

Figure 2: Expression of *H2A* coincides with increase in budding percentages of cells advancing through the cell cycle [taken from ref. 10]**.**

cell cycle mapped by determining budding percentages at steady intervals. It is clearly visible that limiting the amount of galactose leads to a delayed onset of the S-phase (10).

Regulation of cell cycle progression

Progression through the G1 phase is dependent upon the presence of the G1 cyclins Cln1p, Cln2p and Cln3p. Neither of these proteins is essential for passing Start, but deletion of all three genes results in a G1 arrest with unbudded cells and unreplicated DNA (11, 12). Cln1 and Cln2 share a 75% homology at the amino acid level, Cln3p is only 20 to 25% homologous to Cln1p or Cln2p. Unlike *CLN1* and *CLN2*, which are expressed in late G1 phase, transcription of *CLN3* is hardly cell cycle regulated but has a transient increase during early G1 phase. The protein Cln3 appears present throughout the cell cycle (13-15). The kinase activity of Cln3-Cdc28 is approximately 20 fold lower than that of Cln2-Cdc28, when normalized to Cln protein level and Cln2p is about five-fold more abundant when it was expressed from the *CLN3* promoter compared to Cln3p expressed from its own promoter (16). Another feature showing that Cln3p has different functions than Cln1p and Cln2p is the fact that in the absence of *CLN3*, *BCK2* is required for growth, while it is not required in the absence of both *CLN1* and *CLN2*. Bck2 functions in the same pathway as the serine/threonine phosphatase Sit4p, which is also required for accumulation of *CLN1* and *CLN2* but not *CLN3* mRNAs (see below for more details about Sit4 and regulation of the cell cycle). Bck2p is a protein with unknown working mechanism that can complement the cell lysis defect of mutations in the Pkc1 cell integrity pathway (see below for more information about the Pkc1 pathway in relation to the cell cycle). The function of Bck2p to induce expression of *CLN1* and *CLN2* depends on SBF and MBF, just like Cln3p. In contrast to Cln3p however, the function of Bck2p is not dependent on Cdc28p. The lethality of a *bck2 cln3*Δ strain is rescued by overexpression of *RME1*, which drives transcription of *CLN2*. This transcription is completely independent on SBF or MBF transcription factors. In the absence of *CLN3*, Bck2 activates transcription of *CLN1* and *CLN2* (17, 18).

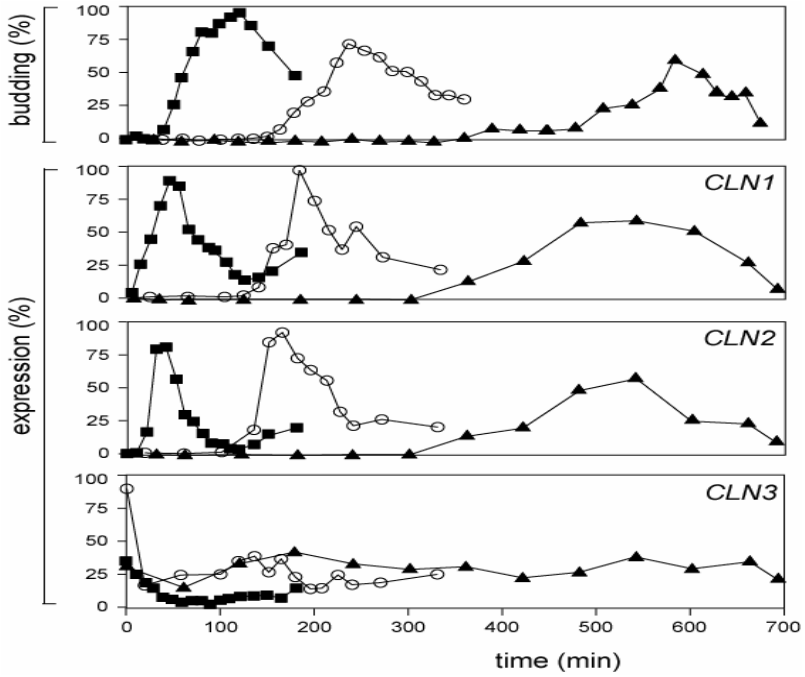

Figure 3: The expression of cell cycle regulating transcripts like *CLN1*, *CLN2* and *CLN3*. Elongation of the G1 phase and therefore the cell cycle was obtained by limiting the galactose feed in a fed-batch system. The duration of the G1-phase of cells growing in 2 % galactose (■) is approximately 65, while the G1-phase of cells administered 41 fmol / cell / hour (○) or 30 fmol / cell / hour (▲) takes 225 respectively 580 min. This figure is taken from ref. 10.

As described above, growing synchronized cells in fed-batch cultures clearly demonstrated that elongation of the G1 phase by low galactose consumption rates is accompanied by a delayed expression of *CLN1* and *CLN2* (Fig. 3), which is always at the end of the G1 phase (10). However, a *cln1 cln2* mutant is viable. The reason for this is that other cyclins, such as the cyclins Clb5p and Clb6p or Pcl1p and Pcl2p, can take over their function albeit with less optimal efficiency. Cells deleted for c*ln1 cln2 clb5 clb6* and *cln1 cln2 pcl1 pcl2* are not viable and they arrest in G1 phase as large, unbudded cells with unreplicated DNA (19). Cln1p and Cln2p have overlapping functions in promoting Start-related events. These events include budding, DNA replication and inhibition of Clb degradation (12, 20).

The transcription factors SCB binding factor (SBF) and MCB binding factor (MBF) are activated by Cln3p. SBF consists of the DNA-binding protein Swi4 and the regulatory protein Swi6, and binds the repeated upstream regulatory sequence CACGAAA, called SCB for Swi4,6-dependent cell cycle box. Swi4 binds the SCB sequence via an N-terminal helix-turn-helix DNA binding domain. The two carboxyterminal parts of Swi4p and Swi6p interact and by this, Swi6p regulates SBF function (21). *SWI4* expression was demonstrated to occur approximately 10-20 minutes before *CLN1*and *CLN2* expression when cells are grown at high growth rates, but this time interval increased strongly during very low growth rates in the above-mentioned fed batch culture conditions (10).
MBF consists also of Swi6p, but cooperates with the DNA binding protein Mbp1 that binds the upstream regulatory sequence ACGCGT, or MCB for Mlu1 cell cycle box (21).

More than 200 genes have a maximal expression late in G1 phase and most of them have one or more SCB or MCB elements in their upstream sequences. Genes with SCB elements in their promoter include *CLN1* and *CLN2*, and the Pho85-associated cyclins *PCL1* and *PCL2*. These four cyclins are together with their Cdk's required for the G1-S transition. Other genes that are activated include genes involved in cell wall biosynthesis and budding (22). MBF activated genes are predominantly involved in DNA repair and DNA replication (23).

Deletion of *SWI4* results in defects in budding (24). Deletion of *CLN3* and *SWI4* results in near lethality (25). Cells from a strain in which both *swi4* and *mbp1* are deleted arrest in G1 phase. Cells deleted for *swi4* and *swi6* show a similar phenotype, probably because Mbp1p is inactive in the absence of *SWI6*. *swi6* and *swi6 mbp1* mutants are viable, probably because Swi4p acts as a transcription factor in the absence of Swi6p, albeit at lower capacity. Swi6p is localized to the cytoplasm during most of the cell cycle, but is nuclear during G1 phase. This nucleo-cytoplasmic shuttling is regulated by phosphorylation of Ser160, which is close to the nuclear localization signal of Swi6p. Phosphorylation of this serine residue is thought to inhibit the NLS and prevent nuclear localization during S to M phase transition (26).

SBF regulation via the subcomponent Swi6 occurs via Cln3p. The regulation of cell size, pheromone sensitivity, S phase entry and budding by Cln3p depends on Swi6p, although the consensus phosphorylation sites for Cdc28p are not involved and stable complexes between Swi6p and Cln3p were not detected (27).

During late G1 phase, Cln1p and Cln2p levels are higher than Cln3p levels and the Cdc28p kinase activity associated with Cln3 is lower. Substrate specificity also seems to differ between Cln1p and Cln2p on one hand and Cln3p on the other hand. The idea is that the role of Cln1/Cln2-Cdc28 activity is to target Sic1p for degradation allowing Clb5p and Clb6p to associate with and activate Cdc28p kinase activity, while the main function of Cln3-Cdc28 is to activate transcription of *CLN1* and *CLN2* (16). Besides protein levels and substrate specificity for Cdc28p, the localization of cyclins is also important for proper regulation of Cdc28p function/activity. The localization of Cln2p is mainly cytoplasmic, while that of Cln3p is nuclear in large, budded cells. In the cytoplasm, Cln2p is localized to sites of budding. Shuttling of Cln2p between the nucleus and the cytoplasm is important for correct functioning of the protein. Correct functioning of Cln3p requires a nuclear localization. This localization is achieved by a bipartite nuclear localization signal and is not dependent on interaction with Cdc28p. Mutations in Cln2p or Cdc28p that abolish the interaction, result in loss of nuclear localization (28, 29). While Cln1- and Cln2-Cdc28 kinase activity is regulated by the expression of *CLN1* and *CLN2*, the kinase activity of Cln3-Cdc28 is regulated by the carbon source. The activity is highest in cells growing on glucose and lowest in cells growing on non-fermentable carbon sources such as ethanol. Also, *CLN3* mRNA levels are regulated by carbon source and the Ras-cAMP pathway positively regulates Cln3 protein and activity levels. In addition, the Ras-cAMP pathway seems to affect positively *CLN1* and *CLN2* expression as inhibition of the pathway leads to a G1-arrest that is equal to that seen in nutrient starved cells. Depletion of cAMP from the medium does hardly influence *CLN3* mRNA levels, while it decreases *CLN1* and *CLN2* mRNA levels and overexpression of *CLN3* eliminates the requirement for cAMP. Deletion of *cln3* inhibited induction of *CLN1* and *CLN2* mRNAs by cAMP (30).

Inhibition of the TOR pathway by rapamycin leads to a G1 arrest with large unbudded cells that do not express the G1 cyclins. This G1 arrest is however a secondary result as ectopic expression of *CLN2* does not rescue the G1-arrest (31). Binding of Whi3p, a *CLN3*-RNA binding protein also decreases progression through the cell cycle in a dose-dependent manner with overexpression of *WHI3* causing G1 arrest (32). *whi3*Δ cells enter S phase at a size that is approximately 25% smaller than wild type cells and expression of G1/S transition genes starts at smaller cell sizes. The Cln3 protein and phosphorylation levels were not affected in these *whi3*Δ mutants. A possible explanation for this is that Whi3p restricts Cln3p at cytoplasmic foci and thereby inhibits activation of SBF and MBF by Cln3-Cdc28 (33).

When cells have passed Start, the Clb5 and Clb6 cyclins interact with Cdc28p, each at a different time, but their expression overlaps. DNA replication is induced by both Clb5p and Clb6p followed by induction of mitosis by Clb1-Clb4 (34, 35). Late in the G1 phase, the transcription factor MBF initiates expression of *CLB5* and *CLB6* (36, 37). During the S phase, *CLB3* and *CLB4* are expressed and followed by *CLB1* and *CLB2* during the G2 phase.

The role of Clb5p and Clb6p in DNA replication appears to be similar, but not identical. When *clb5* is deleted, DNA replication is initiated normally, but it takes cells twice as long to complete replication. When clb*6* is deleted, no changes in onset and duration are observed, but when both *clb5* and *clb6* are deleted, the phenotype is similar to that of *CLB5* mutants: S phase is delayed and bud formation and DNA replication occur no longer at the same time. So, it seems that Clb6p has a minor role that is not yet elucidated (34, 38). When cells are deleted for *clb1*, *clb2*, *clb3* and *clb4*, they normally initiate and finish DNA replication but fail to form bipolar mitotic spindles and these cells arrest in G2 phase. The activation of Cdc28p kinase by Clb1-4 is required to stop polarized bud growth. Early spindle assembly during the S phase is due to Clb3p and Clb4p, while the elongation is under control of Clb1p and Clb2p (35, 39).

One of the targets of Clb-Cdc28 kinase activity concerns the transcription factor *SWI5*. Swi5p is nuclear during G1 phase, while it accumulates in the cytoplasm before the end of anaphase. This accumulation is dependent on Cdc28p-dependent phosphorylation of its nuclear localization signal. Inactivation of Clb-Cdc28 kinase activity at the end of mitosis might allow nuclear localization, where Swi5p can activate transcription of a number of genes, including that of the Cdk inhibitor *SIC1*. *SWI4* might also be regulated by Clb-Cdc28 kinase activity, because repression of SBF-regulated genes depends on *CLB1*, *CLB2*, *CLB3* and *CLB4*, and association of Swi4p with Clb2p can be detected in cells that are arrested in mitosis (40, 41).

Besides the essential Cdc28p, the Cdk Pho85p plays important roles in cell metabolism and division but is not directly involved in the cell cycle. Cdc28p and Pho85p are 51% homologous to each other. Like Cdc28p, Pho85p interacts with and is activated by multiple cyclins (42). The cyclins Pcl1p and Pcl2p seem to play a role in the regulation of G1 phase, although they are not essential and deletion of both of the genes does not lead to a G1 cell cycle arrest. When *cln1* and *cln2* are deleted however, *PCL1* and *PCL2* become essential, as well as *PHO85*. Expression of *PCL1* and *PCL2* is under control of SBF and is highest at Start. Overexpression of *PCL1* and *PCL2* rescues cell lysis as observed in cells deleted for members of the *SLT2* pathway. *SLT2* is the MAP kinase (MAPK) of the *PKC1-SLT2* pathway, also called the cell wall integrity pathway (see below). Over-expression of *CLN1* and *CLN2* does not suppress this defect, indicating that although the genes can complement each other, they also have different roles (43).

G1 to S transition is negatively regulated by the CLB kinase inhibitor Sic1

Before cells enter S phase, Sic1p has to be ubiquitinated and subsequently degraded. For ubiquitination, Sic1p needs to be phosphorylated at least six different positions at the same time and Cln1/2-Cdc28 and Pcl1/2-Pho85 kinase activity mediate these phosphorylations (44-46). Although six phosphorylations are sufficient to target Sic1p for destruction, it contains nine

potential phosphorylation sites. Seven of these sites are clustered in the first 105 N-terminal residues (46). When *sic1* is deleted, cells suffer from premature DNA replication and genome instability (47). Sic1p does not only inhibit G1 exit, it also promotes G1 entry after mitosis, both by inhibition of Clb-Cdc28 kinase activity (48). The inhibition of Clb-Cdc28 kinase activity at the end of mitosis induces spindle disassembly, formation of two nuclei and cytokinesis (49). Ubiquitination and degradation of the Clb cyclins causes Clb-Cdc28 kinase inactivation (50). *SIC1* transcription increases late in M phase and this increase is dependent on the transcription factor Swi5p. *SIC1* expression is inhibited at the G1 to S transition. When Sic1p is degraded as a result of Cln1/2-Cdc28 kinase activity, the inhibition of Clb5/6-Cdc28 kinase activity is released and DNA replication is initiated. By deletion of *SIC1*, the delay in DNA replication in *cln1 cln2* mutants is suppressed (44-46, 48).

Signal transduction pathways involved in cell cycle regulation.

To maintain a certain mean cell size, the cell has to control the increase in cell volume in relation to cell cycle progression. The initial idea was, that when a critical cell size is reached, the levels of Cln3-Cdc28 increase, Cln3-Cdc28 positively regulates Swi4p, which together with Swi6p induces expression of SBF-regulated genes. Among these genes are *CLN1* and *CLN2*, and Cln1/2-Cdc28 activity then initiates passage through Start (51). However, the cell size appears not to be the critical determinant for passage through Start, because it was demonstrated that cells start to bud at a small size when grown on high galactose levels, and at a larger size when grown on low galactose levels (10). Apparently, proteins or regulatory systems are important for regulation of passage through Start.

In mammalian cells, the MAPK pathway has been demonstrated to play an important role in progression through the G1 phase of the cell cycle (52). This tempted us to suggest that in yeast cells MAPK pathways might also be positively involved in progression of the cell cycle. It has been shown that the Pkc1 pathway is responsible for the phosphorylation of Swi4p and Swi6p

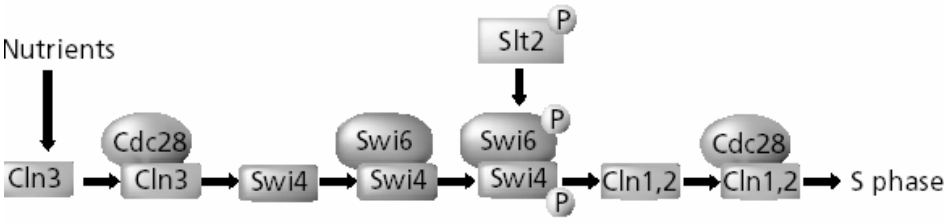

Figure 4: Initial Model of G1 phase regulation by *PKC1-SLT2* pathway. Nutrients induce the transcription of *CLN3* and Cln3 forms a complex with Cdc28. This complex initiates formation of the SBF complex consisting of Swi4 and Swi6. Active Slt2 phosphorylates Swi4 and Swi6, which then induce transcription of *CLN1* and *CLN2*. Cln1 and Cln2 complexed to Cdc28 finally induce progression to S phase. Model after ref. 60.

and this phosphorylation is thought to be the activating step (43). The Pkc1 pathway consists of the upstream kinase Pkc1p, the MAPKKK Bck1p, the MAPKKs Mkk1p and Mkk2p and finally the MAPK Slt2p (53-56). Pkc1p can be activated via Rho1p by several membrane receptors, namely Wsc1-4 and Mid2p, which all seem to signal under different conditions, but also seem to have overlapping functions (57-61). Mid2p is specifically activated when cells are exposed to mating pheromone (58) while *wsc1wsc2wsc3*Δ cells hardly activate the *PKC1-MAPK* pathway in response to heat shock (57). It appeared that Slt2p becomes active just before cells enter the S phase and this provided a clue that members of the *PCK1-SLT2* pathway may positively influence cell cycle progression. This was strengthened by the observation that cells deleted for *SLT2* hardly formed new cells. Furthermore, cells progressing faster through the cell cycle by overexpression of *CLN3* contained more phosphorylated and hence active Slt2p compared to wild type cells, indicating that Slt2p may be one of the downstream targets of Cln3 and it was suggested to be an upstream regulator of Cln1p and Cln2p (figure 4) (62).

However, facts that contradict this suggestion concern the observations that the Pkc1 pathway is activated when cells are exposed to high temperature, hypo-osmotic shock and compounds that interfere with cell wall synthesis. The Pkc1 pathway is also activated in response to pheromone treatment, although this activation is an indirect event due to cell wall changes

induced by activation of the pheromone MAPK pathway (6). Activation of the MAP kinase Slt2p is only partially dependent on Cln1/2-Cdc28 kinase activity, which places Slt2p downstream of Cln1/2-Cdc28 instead of Slt2p being an upstream regulator of Cln1p and Cln2p (24). When cells deleted for *slt2* were grown on 1 M sorbitol, their growth rate was only slightly lower than that of wild type cells grown on 1 M sorbitol and examination of the abundance of Cln1p and phosphorylated Slt2p revealed an earlier appearance of Cln1 than phosphorylated Slt2p (unpublished observations). Furthermore, overexpression of *CLN1* or *CLN2* did not suppress defects of Pkc1ppathway mutants (24, 43). These observations do however not devaluate the role of Slt2p in the G1-S transition, because Slt2p is activated at the G1-S transition at the same time as bud emergence; the growth defect of certain G1-defective *cdc28* alleles is exacerbated by *slt2* mutants and certain *pkc1* alleles and overexpression of *BCK2* partially suppressed the defects of *pkc1* pathway mutants (24). It was also demonstrated that both Slt2p and Cln2p localize to the site of budding (63, 64) although Slt2p is probably not responsible for bud formation as *PKC1* deletion mutants are capable of forming buds (65). During the cell cycle, *CDC*28 is required for timely activation of Slt2p, but during other conditions, *e.g*. exposure to mating factor, activation of Slt2p is independent of Cdc28p (6). The activation of Slt2p by Cdc28p occurs through phospholipase C (PLC). Cdc28p activates PLC, which cleaves phosphatidylcholine to diacylglycerol (DAG) and phospholipid (PL). Pkc1 is known to contain a binding site for DAG, through which the Pkc1 pathway is activated (6, 65). When Slt2p is activated, the expression of *PCL1* and *PCL2* increases, indicating that active Slt2 promotes cell wall biosynthesis, which is important for proper bud formation. Thus the renewed model (figure 7) places Slt2p downstream of Cln1p and Cln2p, but upstream of *PCL1* and *PCL2* and other genes. Overexpression of *SWI4*, *PCL1* or *PCL2* does indeed suppress the growth defect of *slt2* mutants (43).

As Slt2-GFP is localized to bud tips and the nucleus when cells are exposed to mating factor (64, 66), Slt2p may also have a nuclear localization at the end of the G1 phase. Apparently Slt2p functions to activate the transcription factors Rlm1p, which induces genes of cell wall proteins and

enzymes involved in cell wall biosynthesis (67, 68), and SBF which is, next to regulation of the cell cycle, also involved in membrane and cell wall biosynthesis and budding (69). Activated Slt2p has to be dephosphorylated in order to decrease the activity of the Pkc1-pathway. Several phosphatases are known to deactivate Slt2p: i.e. Ptp2p, Ptp3p, Msg5p and Sdp1p (70-72). Upon heat shock and H_2O_2 treatment, the mRNA levels of *SLT2*, *SDP1* and *PTP2* are induced. *PTP3* and *MSG5* mRNA levels did not change upon H_2O_2 treatment and only transiently increases upon heat shock (70). All mRNAs were induced after osmotic stress, but whether the activity of these phosphatases also increases is unknown. These examples indicated that different phosphatases are expressed and activated under different stress conditions.

As mentioned earlier, it is thought that the PKA/cAMP and the TOR pathways are also involved in the cell cycle regulation by regulating protein synthesis rate (73). How these two pathways influence cell cycle progression is not known yet. One of the components of the TOR pathway is Sit4p, a serine/threonine phosphatase that is involved in a number of processes, including the TOR-pathway-regulated nutrient response, regulation of ion homeostasis and intracellular pH and cell cycle regulation (74-76). Cells deleted for *SIT4* exhibit a low growth rate due to an increase in G1 phase duration, depending on a gene called *SSD1*. *SSD1* is a polymorphic gene that can suppress the lethality of *sit4*Δ cells in some laboratory strains, but can not in others (77). This extension of the G1 phase is partly due to the role *SIT4* normally plays in the transcription control of the G1 cyclins *CLN1* and *CLN2*, and in the control of *SWI4* expression. Sit4p is thought to act in the pathway parallel to Cln3-Cdc28, the pathway involving Bck2p, to activate *CLN1* and *CLN2* expression (17, 78). Indirectly, Sit4 is thus required for normal DNA replication. Besides DNA synthesis, Sit4p is also thought to play a role in bud formation. Sit4p is placed upstream of the Pkc1 pathway but downstream of the cell surface receptors Wsc1-4 and Mid2p, and deletion of *SIT4* leads to an up-regulation of the Pkc1 pathway and to an enrichment of G1 cells in the culture (79).

While Sit4p is a positive regulator of cell cycle progression, the phosphatases Ppz1p and Ppz2p inhibit cell cycle progression. Overproduction

of *PPZ1* can rescue the cell lysis defect of mutants of the Pkc1 pathway, but severe overexpression of *PPZ1* results in a delay in the G1 to S transition, as high levels of Ppz1 seem to interfere with budding initiation and DNA synthesis (79, 80).

Another regulator of cell cycle progression, but on the transcription level, is Azf1p, which induces *CLN3* transcription in a glucose concentration-dependent manner, although the mechanism is not yet clear (73).

Deletion of *KNR4*, a gene that encodes for a protein with unknown function, is lethal when members of the Pkc1 pathway are missing, but overexpression of *KNR4* does not rescue the cell lysis phenotype of Pkc1 pathway deletion mutants. It is known that *KNR4* and *BCK2*, which are involved in Cln3-Cdc28 independent gene expression, function in the same genetic pathway, but they must also have separate functions as indicated by deletion studies. When *CLN3* is deleted, not only *BCK2* is required, but also *KNR4* (81, 82). Deletion of *KNR4* deregulates transcription of cell cycle-regulated genes such as *CLN2* and *PCL1* (82).

Relation between the nutrient availability, G_1 phase duration and reserve carbohydrate accumulation

By growing synchronized cells in fed-batch cultures, a tight correlation between nutrient availability and the duration of the G1 phase was observed, which was shown for haploid (83) and diploid cells (10). The cells were cultured under galactose limitation, which was added to the cells at various but constant rates as described above. The results of these studies demonstrated that the relationship between the flux of the growth rate determining nutrient and the length of the G1 phase was bi-phasic (Figure 4). When the galactose consumption rate of haploid cells was decreased from 50 to 20 fmol $cell^{-1}.hr^{-1}$, the G1 phase duration gradually increased from minimally 110 to 160 minutes in length. At consumption rates lower than 20 fmol $cell^{-1}.hr^{-1}$, the G1 phase duration rapidly elongated from 160 to 520 minutes at 14 fmol $cell^{-1}.hr^{-1}$ (83).

Furthermore, these studies also revealed that an increase in the G1 phase duration was accompanied with trehalose and glycogen accumulation

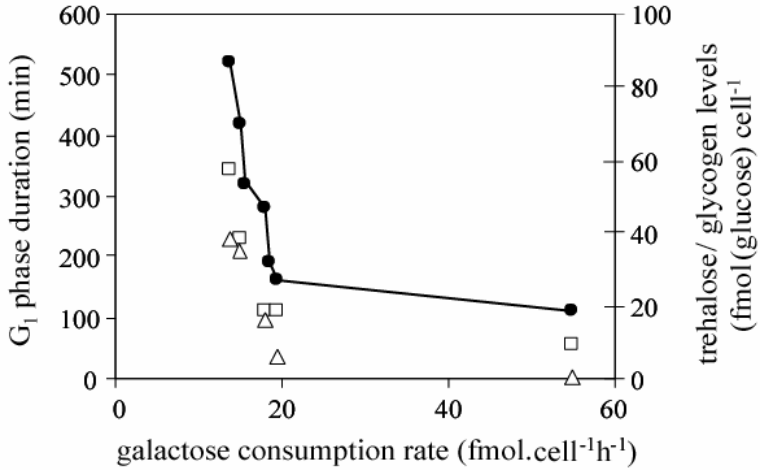

Figure 5. Bi-phasic relation between G1 phase duration, trehalose and glycogen accumulation and the galactose consumption rate. Haploid wild type cells synchronized early in the G1 phase were grown at different galactose consumption rates. The G1 phase duration is defined as the time from inoculation until 50% budding was reached. (●) G1 phase duration (min), (□) glycogen (fmol (glucose) cell^{-1}), (Δ) trehalose (fmol (glucose) cell^{-1}). This figure was taken from (60).

when the galactose consumption rate was lower than 20 fmol cell^{-1}hr^{-1} in haploid cells (Figure 4, (83)), and below 31 fmol cell^{-1}hr^{-1} in diploid cells (10). These studies therefore indicated that accumulation of trehalose and glycogen is related to the length of the G1 phase. Of particular interest were the observations that the bi-phasic relation between length of the G1 phase and galactose consumption rate was not observed in a mutant unable to synthesize trehalose and glycogen (62). These observations suggested that the extreme lengthening of the G1 phase when cells were grown at a low galactose consumption rate was solely due to the fact the trehalose and glycogen were accumulated under these conditions. Thus, besides various signalling proteins, accumulation of reserve carbohydrates are also involved in progression through the G1 phase of the cell cycle.

Proteins involved in reserve carbohydrates metabolism

Several carbon sources may be used as precursors for trehalose and glycogen (Figure 5). Upon transport into the cell, glucose is phosphorylated

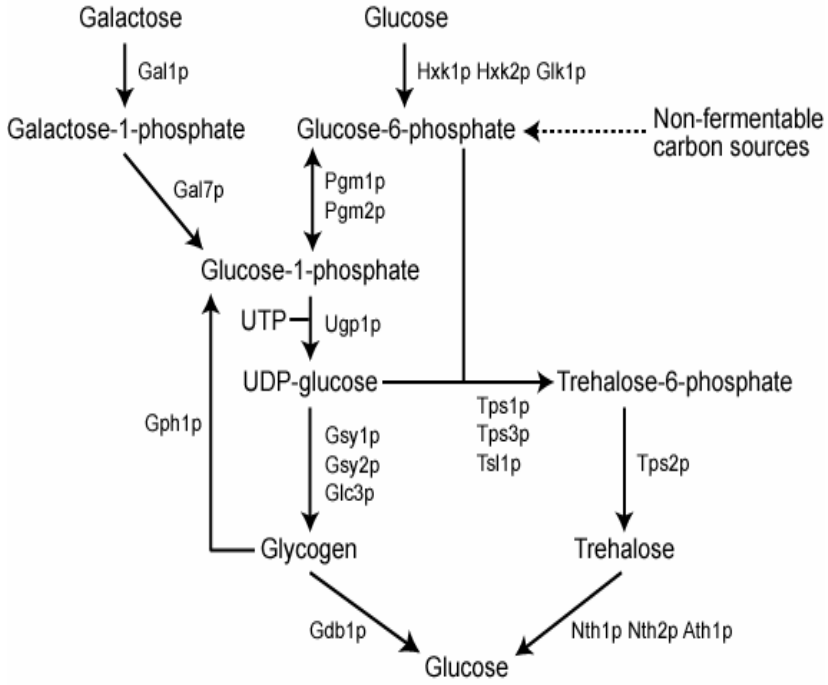

Figure 6. Overview of trehalose and glycogen metabolism in *Saccharomyces cerevisiae*. Many carbon sources can serve as precursors for trehalose and glycogen. The proteins and metabolites that are involved in trehalose and glycogen metabolism are indicated. Tps1p, Tps2p, Tps3p and Tsl1p are present in the trehalose synthase complex. For details see text.

into glucose-6-phosphate by hexokinase (84) and converted into glucose-1-phosphate by phosphoglucomutase, encoded by *PGM1* or *PGM2* (85). Subsequently, glucose-1-phosphate is converted into UDP-glucose by UDP-pyrophosphorylase (Ugp1p, (86)). Galactose may serve as precursor for trehalose and glycogen as depicted in Figure 5 (87). Non-fermentable carbon sources like ethanol or glycerol, which may be ultimately converted into glucose-6-phosphate by gluconeogenesis, can also be used as precursors for trehalose. During conditions that result in accumulation of reserve carbohydrates, the flux towards the precursors of trehalose and glycogen may

be increased by induction of *PGM2* and *UGP1* expression, as well as regulation of the enzymatic activities of the enzymes (85, 86, 88).

As shown in Figure 5, trehalose is synthesized by the conversion of glucose-1-phosphate or UDP-glucose into trehalose-6-phosphate, which is catalyzed by trehalose-6-phosphate synthase, encoded by *TPS1* (89, 90). Subsequently, trehalose-6-phosphate phosphatase (Tps2p) mediates the conversion of trehalose-6-phospate into trehalose and free phosphate (91, 92). Tps1p and Tps2p are part of a large protein complex called the trehalose synthase complex, which also contains the products of the *TSL1* and *TPS3* genes. Tsl1p and Tps3p are regulatory components that probably function to stabilize the complex (89, 93, 94). Trehalose can be degraded by the action of two types of trehalases. The first type, encoded by the *NTH1* gene (95, 96), is a cytoplasmic protein (97), which has its maximal activity at pH 6.8 – 7.0 and is therefore called neutral trehalase (98). *NTH2* encodes a protein with 77% similarity to Nth1p, although the cellular role of Nth2p remains unclear (99). The second type of trehalase is found in vacuoles (97) and is encoded by *ATH1* (100). The enzyme is optimally active at pH 4.5 – 5.0 and therefore designated as acidic trehalase (98). Synthesis of glycogen involves initiation, elongation and ramification steps, which are mediated by the enzymes encoded by *GLG1*, *GLG2* and *GLC3* (101-103). Glycogen degradation is mediated by the proteins encoded by *GPH1* and *GDB1* (104, 105).

The role of Tps1p

Yeast cells deleted for *tps1* display a variety of phenotypes, of which the inability to grow on rapidly fermentable carbon sources like glucose is the most profound (106). Upon addition of glucose, *tps1* deletion mutants hyperaccumulate glycolytic intermediates, and have reduced intracellular levels of inorganic phosphate and ATP (107, 108). Reduction of ATP levels suggests an imbalance between the upper part of glycolysis, in which ATP is consumed, and the lower part of glycolysis, where ATP is generated. Mutations that reduce sugar transport reduce the glucose-growth defect, indicating an excessive sugar influx into glycolysis *tps1* deletion mutants (109, 110). Deletion

of *hxk2* in the *tps1* deletion mutant, thereby lowering the glucose phosphorylation rate, results in regain of growth on glucose (109, 111). These results imply that the system responsible for trehalose production is also important for the control of glucose influx in glycolysis (111, 112). It was proposed that Tps1p acts as a direct regulator of glucose transport and phosphorylation, in conjunction with a sugar carrier and a sugar kinase to form the "general glucose-sensing complex" (89, 94, 106, 113). The observation that Tps1p may exists both as a free form and as a component of the trehalose synthase complex supports the suggestion that Tps1p might exhibit additional regulatory functions (94). Furthermore, these observations also suggest that a link might exist between glucose transport and accumulation of trehalose. Glucose, which can be transported by hexose transporters, is used as a precursor for trehalose, during conditions when glucose becomes limited, or during conditions when an extra demand for trehalose is required to withstand harmful conditions. This glucose may be gained by lowering the amount of glucose destined for glycolysis, or by increasing the uptake of glucose, for example by increasing the amount of active glucose transporters. A system that couples glucose transport directly to accumulation of trehalose during conditions of slow growth could be beneficial for the yeast cell.

The possible role of Hxt5p in trehalose accumulation

In *S. cerevisiae*, transport of glucose across the plasma membrane is mediated by a multigene family consisting of 20 genes, including *HXT1-HXT17*, *GAL2*, *SNF3* and *RGT2*, encoding Hxt proteins, (114-117). Individual expression of *HXT1-4* and *HXT6-7* in the MC996 background strain, which was deleted for *HXT1-7* (*hxt* null mutant), resulted in regaining the ability to grow on glucose. Over-expression of *HXT5* or *HXT8-17* (except *HXT12*, which probably encodes a pseudogene) individually in the CEN.PK background strain deleted for *hxt1-17* and *gal2* restored growth on glucose, indicating that Hxt5p, Hxt8-11p and Hxt13-17p are also able to transport glucose (118). Therefore, *HXT1-4* and *HXT6-7* were initially thought to encode the major Hxt proteins (119). However, it was shown later that Hxt5p also exhibits glucose transport

capacity (120, 121). Expression of the major *HXT* genes is mainly regulated by extracellular the glucose concentration (115).

It appeared that expression of one of the remaining hexose transporters is regulated in a completely different manner as compared to the major *HXT* genes. *HXT5* expression was not regulated by the extracellular glucose concentration, but by the growth rate of yeast cells. Expression of *HXT5* was specifically induced when the growth rate of cells decreased, and was maximally induced when glucose was depleted from the growth medium. Furthermore, during exponential growth on fermentable carbon sources other than glucose, like galactose, maltose, mannose or sucrose, *HXT5* was not expressed, but 24 hours after inoculation, during the stationary phase, *HXT5* was expressed independent of the carbon source used. *HXT5* was also expressed during growth on the non-fermentable carbon sources ethanol and glycerol, which results in decreased growth rates as compared to growth on fermentable carbon sources. Experiments conducted in nitrogen-limited continuous cultures indicated that *HXT5* expression is indeed regulated by the growth rate of cells, as *HXT5* was only expressed at dilution rates lower than 0.10 h^{-1}, and not at higher dilution rates (122).

Surprisingly, it appeared that the conditions that result in a decrease in the growth rate of cells and thereby in an increase of expression of *HXT5* also resulted in accumulation of trehalose (123). During growth in a nitrogen-limited continuous culture, trehalose was only accumulated at dilution rates lower than 0.10 h^{-1}, concomitant with *HXT5* expression. In glucose-grown batch cultures, only the expression pattern of *HXT5* completely matched the pattern of trehalose accumulation, whereas expression of the major *HXT* genes only partially overlapped the trehalose accumulation pattern. Furthermore, during growth on non-fermentable carbon sources, *HXT5* was also expressed concomitant with trehalose accumulation (123). These observations suggested that Hxt5p plays a role in accumulation of trehalose, specifically during growth on glucose but also during growth on other carbon sources.

Growth rate-regulated gene expression

Expression of genes is regulated by binding of transcription factors to regulatory elements that are present in the promoter of genes. Having established that expression of *HXT5* is regulated by the growth rate of yeast cells, this feature may be used as a tool to determine the mechanism behind growth rate-regulated expression of *HXT5* and perhaps also for expression of other growth rate-regulated genes. It appeared that the promoter of *HXT5* contained 5 putative regulatory elements being two stress responsive elements (STREs, (124-127)), one PDS element (128) and two Hap2/3/4/5p (HAP) complex binding sites (129). The STREs are located at -472 bp and -304 bp relative to the translation initiation site respectively, the HAP2/3/4/5 binding sites at -854 bp and -785 bp respectively and the PDS element at -544 bp, which was determined by computer-assisted analysis of the *HXT5* promoter (130).

Mutational analysis each of these putative elements in the promoter of *HXT5* revealed that the STRE most proximal to the translation initiation site (now to be named STRE1) was extremely important in growth rate-regulated expression of *HXT5* (123). STREs are bound and activated by the transcription factors Msn2p and Msn4p, which may bind to STRE1 in the promoter of *HXT5* and could therefore be the most important transcription factors involved in regulation of *HXT5* expression.

Growth rate-regulated *HXT5* expression is controlled by STRE1 The presence of STREs in the *HXT5* promoter suggests that the transcription factors Msn2 and Msn4p, which are known to bind to STREs (131, 132), are involved in regulation of *HXT5* expression.

In order to act as transcription factors, Msn2/4p have to be present in the nucleus, and it is known that the activity of the cAMP/PKA pathway determines the localization of Msn2/4p (133). The activity of the cAMP/PKA pathway is highly correlated to the availability and quality of the nutrients, and thereby to the growth rate of cells. The activity of the cAMP/PKA pathway is high when cells grow in a medium containing a high amount of fermentable carbon sources, resulting in high growth rates of cells (113, 134). During these

conditions, the transcription factors Msn2/4p are present in the cytoplasm. The activity of the cAMP/PKA activity decreases when the medium becomes deprived from fermentable carbon sources, for example when cells enter the diauxic shift, resulting in a decrease of the growth rate of cells and Msn2/4p translocation to the nucleus. Also during stress conditions, like growth at increased temperatures or osmolarity, the growth rates of cells decrease and Msn2/4p become localized in the nucleus (133). When Msn2p and Msn4p are present in the nucleus, expression of genes that are enriched in STREs in their promoter region is induced (135, 136).

Thus, in addition to regulation of expression of *HXT5*, the cAMP/PKA pathway might regulate expression of a variety of genes in response to decreased growth rates of cells, predominantly through inhibition of STRE-driven gene expression at high growth rates. This was concluded from the observation that expression of *HXT5* was co-induced with a variety of other genes, as determined by DNA microarray analysis, during a variety of conditions that result in a decrease in the growth rate of cells. These studies include addition of salt (137-139), cell damaging agents (140, 141) or ethanol (142). Also, Gasch *et al* (143) described the family of environmental stress response (ESR) genes, that corresponds to a cluster of genes that have similar expression patterns under a variety of stress conditions, for example heat shock, osmotic shock, growth into the stationary phase and nitrogen depletion. All these conditions are again known to reduce the growth rate of yeast cells, and result in induction of *HXT5* expression. The clusters of genes were characterized by the presence of a high amount of STREs in the promoters of these induced genes. Apparently, the conditions that result in decreased growth rate of the yeast cells are associated with decreased activity of the cAMP/PKA pathway and expression of STRE-regulated genes. Therefore, expression of the subset of genes, which is regulated by growth rates of cells, may be specifically controlled by the cAMP/PKA pathway in *S. cerevisiae*.

Conclusion

Clearly, the growth rate is dependent on the duration of the cell cycle and thus of the length of the G1 phase. The accumulation of the reserve carbohydrates is proven to be both a feature and a determinant of an elongated G1 phase as this is accompanied by accumulation of both glycogen and trehalose in wild type cells. A mutant unable to synthesize these carbohydrates does not elongate its G1 phase at low galactose fluxes. Accumulation of trehalose is shown to coincide with expression of the glucose transporter *HXT5*, and a role of Hxt5 in trehalose accumulation is suggested, also because deletion of *HXT5* results in lower trehalose levels. The cAMP-PKA signal transduction pathway might be involved because this pathway regulates genes containing STREs in their promoter, like *HXT5* does. The cAMP-PKA pathway might also regulate cell cycle progression, as *CLN1* and *CLN2* mRNA levels for example are positively regulated by cAMP and both Cln3 protein levels as activity levels are stimulated by cAMP. cAMP us thus a positive regulator of early G1 phase.

A possible regulator of late G1 to early S phase progression is the *PLC1-SLT2* pathway. Deletion of components of this pathway does not affect progression through early G1, but cells lyse because of a weakened cell wall. Overexpression of late G1 genes such as *PCL1* or *PCL2* or the transcription factor *SWI4* relieves the cell lysis defect. The activation of the *PKC1-SLT2* pathway during the cell cycle is known to be partially dependent on Cdc28 and the model presented in figure 7 emerged. Shortly, nutrients stimulate the formation of the Cln3-Cdc28 complex via the cAMP-PKA pathway. This induces transcription of *CLN1* and *CLN2* via the SBF transcription complex. Cln1,2-Cdc28 complexes induce S phase entry both via degradation of Sic1p and via activation of the *PKC1-SLT2* pathway. Activated Slt2p induces transcription of cell wall genes and inhibits simultaneously transcription of cell cycle genes. A sufficiently strong cell wall induces Sit4 activation, which down-regulates the *PKC1-SLT2* pathway and thus expression of cell wall genes. This feedback secures a tight control of progression through G1 and S phase on one hand and the right composition of the cell wall on the other hand.

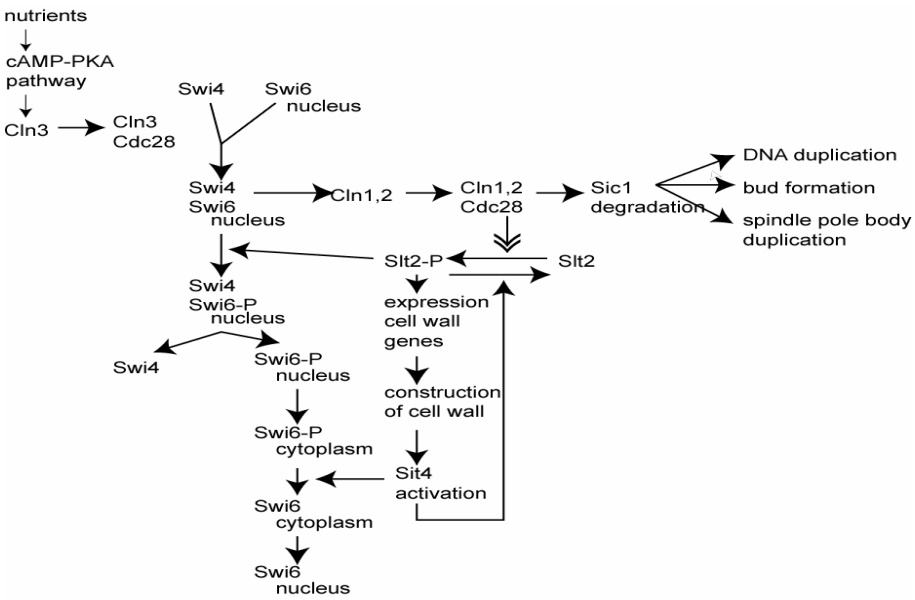

Figure 7: Current model of *PKC1-SLT2* pathway involvement in the cell cycle. Nutrients induce transcription of *CLN3* via the cAMP-PKA pathway. Cln3-Cdc28 induces formation of the Swi4-Swi6 complex in the nucleus. This complex induces transcription of *CLN1* and *CLN2*. Cdc28 complexes to Cln1 or Cln2 regulate Sic1 degradation and thereby DNA replication, bud formation and spindle pole body duplication. Cln1,2-Cdc28 also induces activation of the *PKC1-SLT2* pathway and activated Slt2 phosphorylates Swi6. Phosphorylation of Swi6 by Slt2 leads to inhibition of the nuclear localization signal and the Swi4-Swi6 complex falls apart. Swi6 relocates to the cytoplasm, and Swi4 alone induces transcription of cell wall genes. A correct assembled cell wall leads to activation of Sit4, which inhibits the *PKC1-SLT2* pathway. This leads to inhibition of Swi6 phosphorylation and Swi6 is again capable of entry into the nucleus. Via this feedback mechanism, the production of the cell wall is kept in pace with progression through G1 phase and entry into S phase.

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Chapter 3

Slt2p is activated during the G1 phase in *Saccharomyces cerevisiae* and is required for proper cell cycle progression

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Abstract

The use of cells synchronized by elutriation in combination with fed batch culturing enables monitoring of cell cycle progression without disturbance of cell cycle progression by chemicals or mutations. Culture of elutriated, early G1 phase cells in fed batch cultures with different feeding rates revealed a biphasic relationship between the length of the G1 phase and the rate of galactose addition. Above a switch point, the length of the G1 phase was slowly increased when the flux of galactose was decreased; below this switch point, the length of the G1 phase was sharply increased when the flux of galactose was decreased. In this study we show that this biphasic relationship also exists for fed batch culturing with different glucose rates and that the biphasic relationship between nutrient availability and the length of the G1 phase is dependent on the accumulation of trehalose and glycogen.

In order to place the events at the correct stage of the cell cycle, the protein patterns of Cln1p, Cln3p, Swi4p and Swi6p were monitored. The levels and patterns of these proteins appeared to be dependent on the length of the G1 phase, while only minor differences in protein expression patterns arose from culture on galactose instead of glucose.

Swi6p was phosphorylated at the end of the G1 phase. The phosphorylation of Swi6p is dependent on the activity of the MAPK Slt2p. Here we show that Slt2p is phosphorylated in a cell cycle dependent manner and that this corresponded with Swi6p phosphorylation during the cell cycle. Slt2p phosphorylation is restricted to mid G1 and S phase during short cell cycles. Upon G1 phase elongation, Slt2p is phosphorylated at mid G1 but not dephosphorylated after S phase entry. Deletion of *SLT2* resulted in severe cell cycle elongation. This is comparable with inhibition of MAPK activity in mammalian cells and this is addressed in the discussion.

Introduction

The main cell cycle control point is located in the G1 phase and is called 'Start' in the baker's yeast, *Saccharomyces cerevisiae*. This checkpoint Start is the point where the environmental signals are integrated and the

decision is taken for cell cycle progression (1-3). Once a cell has passed Start, it has committed itself to complete the entire cell cycle before it can arrest the process. One of the environmental factors essential for cell cycle progression concerns nutrient availability. The flux of nutrients supplied to cells has been shown to change the length of the G1 phase, while S, G2 and M phase are kept constant (4, 5). Our group has previously demonstrated a biphasic relationship between the availability of galactose and the length of the G1 phase in both diploid and haploid cells (6, 7). Elutriated, synchronous early G1 phase cells were cultured in fed batch cultures and provided with determined fluxes of galactose. The length of the G1 phase was relatively short with a duration of 110 minutes and increased only slowly to 160 minutes when the galactose flux was lowered from 55 fmol $cell^{-1}$ h^{-1} to 18 fmol $cell^{-1}$ h^{-1}. The galactose flux of 18 fmol $cell^{-1}$ h^{-1} was a switch point as culturing of the early G1 phase cells with a flux less than 18 fmol $cell^{-1}$ h^{-1} resulted in a much larger increase in G1 phase duration from 160 minutes at 18 fmol $cell^{-1}$ h^{-1} to 520 minutes at 14 fmol $cell^{-1}$ h^{-1}. This G1 phase elongation was accompanied with the accumulation of the reserve carbohydrates trehalose and glycogen (6, 7).

The mRNA expression of the G1 phase cyclins and of the transcription factors *SWI4* and *SWI6* has been studied with this system of elutriation and fed batch culturing with determined amounts of galactose (6). Cln3 in complex with Cdc28 induces the transcription of the other two G1 cyclins, *CLN1* and *CLN2* via the SBF transcription factor complex, which consists of *SWI4* and *SWI6* (8, 9). The expression of *CLN3* is comparable between different G1 phase lengths. *CLN1* and *CLN2* expression are rapidly expressed in cells with a short G1 phase, their expression is delayed in cells with an elongated G1 phase. In both situations, the expression of *CLN1* and *CLN2* precedes bud formation with 20 to 50 min. In this same fed batch system, the expression of the transcription factors *SWI4* and *SWI6* was also monitored. *SWI4* is transiently expressed in cells with a short G1 phase with a peak almost simultaneously with *CLN1* and *CLN2* expression. During an elongated G1 phase, *SWI4* expression increases slowly and is kept constant from mid G1 phase onwards. The correlation between *SWI4* and *CLN1*/*CLN2* expression is thereby lost upon G1 phase elongation. SWI6 is in continuously expressed in cells with a

short G1 phase. In cells with an elongated G1 phase, *SWI6* was expressed at a constant level, except for a peak in expression prior to S phase entry (6).

Swi6 is the regulatory component of the SBF complex, while Swi4p is the DNA binding protein (9-14). Swi4p is localized in the nucleus in all stages of the cell cycle, but Swi6p shuttles between the nucleus and the cytoplasm (15, 16). During the S, G2 and M phase, Swi6 is phosphorylated on its serine 160 residue and localized in the cytoplasm. In the G1 phase, unphosphorylated Swi6p is localized in the nucleus and able to support SBF regulated transcription (17). The proteins Cln1p and Cln2p bind Cdc28p and the activity of the Cln1-Cdc28 and Cln2-Cdc28 complexes induce bud formation and facilitate the initiation of DNA replication (8, 18-21). The influence of Cln1-Cdc28 and Cln2-Cdc28 on bud formation might be executed via the mitogen activated protein kinase (MAPK) Pkc1-Slt2 pathway. This signal transduction cascade is involved in the maintenance of cell wall integrity and starts with protein kinase C1 (Pkc1). Pkc1p is activated by its upstream membrane-receptors and by Cdc28p proteins, which are probably bound to Cln1 and/or Cln2 proteins (22-25). Bck1p is downstream of Pkc1p and upstream of the redundant kinases Mkk1p and Mkk2p. The most downstream kinase of this cascade is Slt2p (26-29). Deletion of members of this cascade, alone or in case of the Mkk's together, leads to cell lysis due to cell wall defects. These defects can be relieved by addition of 1M sorbitol to the medium, which relieves the cell wall stress caused by the osmolarity differences between the medium and cell interior (27, 28, 30, 31). The MAPK Slt2p directly influences the transcription factors Rlm1p and SBF (32, 33). Slt2p regulates the phosphorylation of Swi6p, also on the serine residue that controls Swi6p localization (17, 32, 33). The nuclear export of Swi6p enables Swi4p to induce transcription of cell wall genes, which is necessary to allow growth of the developing bud. The activity of Slt2 could therefore determine whether cells progress through the cell cycle.

The MAPK signal transduction pathways are preserved from yeast to mammals and are also present in plants (34, 35). The MAPK cascade is known to be involved in cell cycle progression in various species during both G1, G2 and M phase (36-38). MAPK activity is associated with reactivation of

quiescent (G0) cells and with the G1/S transition (39-42). The level of activity of the MAPK cascade controls the progression through the cell cycle. Both too high and too low levels of activity cause cell cycle arrest (reviewed in (43)). The yeast MAPK cascade is also involved in both G1 and G2 phase progression and the homology between yeast and mammalian MAPK is reflected in the similarities of the processes in which the MAPKs are involved. For example, both the yeast Pkc1 pathway and the mammalian MAPK cascade are involved in G1 phase regulation and during the M phase in spindle pole body formation or monitoring of its status (32, 38, 40, 44).

Although the above described yeast genes and proteins are all involved in G1 phase progression, most of this very valuable knowledge is obtained with experimental setups that used chemicals, temperature sensitive mutants, deletion mutants or tagged proteins. In contrast to these artificial methods, the use of an elutriator gives rise to a highly synchronized population of early G1 phase cells that can be studied without interference of chemicals or redundancy mechanisms (45-47). Culturing of these elutriated, early G1 cells with the use of a fed-batch system provides a method that enables the manipulation of the G1 phase length and close monitoring of cell cycle progression with a minimum of interventions. Because of the homology between roles of mammalian and yeast MAPK during the cell cycle (*e.g.* (32, 38, 40, 44)), we explored the role of *SLT2* in cell cycle progression. To properly place the activity of Slt2p during the G1 phase, we first determined the progression through the G1 phase by examination of the protein expression patterns of Cln1p, Cln3p, Swi4p and Swi6p. Our results indicate regulated protein expression patterns of the G1 cyclin Cln1p, which was dependent on the length of the G1 phase and the carbon source provided. In contrast, Cln3p protein levels were similar for all conditions tested. The transcription factor components Swi4p and Swi6p were influenced in both amount and timing of expression by the length of the G1 phase. Swi4p expression was higher during short G1 phases than during elongated G1 phases, and was similar for glucose and galactose grown cells. The Swi6p levels were different for glucose and galactose cultured cells during short G1 phases, but similar during elongated G1 phases. Slt2 became phosphorylated at mid G1 phase of 105 minutes and

disappeared after S phase entry. In a G1 phase of 420 minutes, Slt2p was phosphorylated also at mid G1 phase but remained present after S phase entry. The phosphorylation of Slt2p is therefore dependent on the length of the G1 phase. Deletion of *SLT2* revealed that the activity of Slt2p is required for proper cell cycle progression.

Materials and methods

Strains, media and growth conditions

The *S. cerevisiae* strains used in this study are shown in Table 1. Strain CEN.PK 113-7D was used as wild type in all experiments. Cells were grown at 30°C at 180 rpm in a shaking incubator (New Brunswick Scientific, Edison, USA) in 0.67% (w/v) yeast nitrogen base without amino acids (Difco, Sparks, MD, USA) and a concentration or flux of the carbon source as indicated in the text. If necessary, the appropriate amino acid was added to the medium at 20µg/ml.

Table 1: List of strains used.

Yeast strain	**Genotype**	**Source**
CEN.PK 113-7D (K43)	MAT**a** *SUC2 MAL2-8^c*	P. Kötter (Frankfurt, Germany)
SCU10	*MAT***a** *SUC2 MAL2-8c* MEL tps1::TRP1 gsy1::LEU2 gsy2::URA3	Sillje et al., 1999 (6)

Elutriation and fed batch culturing

Elutriation and subsequent fed batch experiments using glucose or galactose as carbon source were performed as described (6, 7, 48, 49) with some modifications. Cells were grown over-night in YNB 2% galactose and collected by centrifugation at an OD_{600} of 0.5. The cell pellet was resuspended in the same medium and the cells were sonicated and loaded into the elutriation chamber of the elutriator rotor JE-6B (Beckman Coulter, Inc.) and incubated at 30°C. Small, new-born daughter cells were washed out of the elutriator with a combination of centrifugal force and flow rates and collected on ice, while the larger mother cells and budded cells remained in the

elutriation chamber. After centrifugation, the early G1 phase cells were resuspended in YNB medium containing 0.15 mM galactose. Dependent on the experiment, the rate of glucose or galactose addition to the cells was adjusted with a pump.

Analysis of cell cycle parameters

The cell size was monitored with an electronic particle counter (Coulter MultiSizer II, Beckman Coulter, Inc., Fullerton, CA, USA). Cell concentrations were calculated by Coulter® Multisizer AccuComp® (Beckman Coulter, Inc.) with the use of the amount of sample measured. To determine G1 phase progression, the percentage of budded cells was determined by microscopical analysis of at least 200 cells (6, 49).

Western Blot analysis

Equal amounts of cells were collected by centrifugation, washed with ice-cold water and stored at -20°C until use. RIPA buffer (150 mM NaCl, 20 mM Tris-HCl pH 7.5, 1 mM EDTA, 1% SDS and 0.1% Triton X-100) complemented with protease (Complete, Roche Diagnostics, Basel, Switzerland) and phosphatases (NaF, 50mM) inhibitors was used as lysis buffer. After SDS-PAGE, the proteins were transferred to PVDF membranes (Roche). For detection, the membranes were blocked in PBS (137 mM NaCl, 8 mM Na_2HPO_4, 2.7 mM KCl, 1.5 mM KH_2PO_4), 0.1% Tween-20, 3.4% Protifar Plus (Nutricia, Zoetermeer, the Netherlands) or TBS, 0.1% Tween-20, 5% BSA (Sigma Chemical Company, St. Louis, MO, USA) and subsequently incubated with antibody Sc6690 (goat-anti-Cln1p antibody), Sc6694 (goat-anti-Cln3p antibody), Sc6736 (goat-anti-Swi4p antibody, Sc6736 (goat-anti-Swi6p antibody), all obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA) or CST9101 (rabbit-anti-phospho-p44/p42 MAPK (Thr202/Tyr204) antibody (Cell Signaling Technology, Inc, Danvers, MA, USA) which detects phosphorylated Slt2p (50)). The antibodies obtained from Santa Cruz were used at a concentration of 4 µg/ml, while the other antibodies were used at a dilution of 1:1000. Anti-phospho p44/p42 antibody was diluted in TBS, 0.1%

Tween-20; all other primary antibodies were diluted in PBS, 0.1% Tween-20. After three washes of ten minutes each with PBS, 0.1% Tween-20, 3.4% Protifar Plus or TBS, 0.1% Tween-20, the primary antibody was detected with peroxidase-conjugated donkey-anti-goat or peroxidase-conjugated goat-anti-rabbit (Jackson Immunoresearch Immunoresearch Antibodies Laboratories, Inc., Westgrove, PA, USA). All secondary antibodies were used at a concentration of 40 ng/ml in TBS, 0.1% Tween-20 or PBS, 0.1% Tween-20, 3.4% Protifar Plus. After three washes of ten minutes each with PBS, 0.1% Tween-20, 3.4% PPotifar plus or TBS, 0.1% Tween-20, the proteins were visualized with Enhanced Chemiluminescence (Renaissance, NEN Life Science Products, Inc., Boston, MA, USA).

Results

Relation between the length of the G1 phase and flux of carbon source

Growth of synchronized early G1 phase cells with galactose revealed a biphasic relationship between the length of the G1 phase and the flux of galactose provided to these cells ((6, 7) and figure 1A). At 55 fmol galactose cell^{-1} h^{-1}, the G1 phase was 105 minutes. The G1 phase had increased slowly to

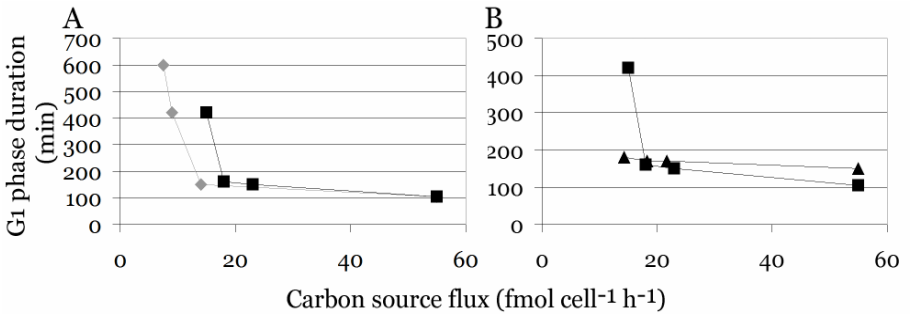

Figure 1. Relation between the duration of the G1 phase and the flux of carbon source. Synchronous, early G1 phase wild type (♦, ■) and tps1Δgsy1Δgsy2Δ (▲) cells were synchronized by centrifugal elutriation and grown at different glucose (♦) or galactose (■,▲) consumption rates as described in materials and methods. The length of the G1 phase was defined as the time from initiation of carbon source addition until 50% of the cells had formed a bud (6).

160 minutes at 18 fmol galactose $cell^{-1}$ h^{-1}. However, the G1 phase rapidly elongated to 420 minutes when the galactose flux was decreased to 15 fmol $cell^{-1}$ h^{-1}. Similar results were obtained when glucose was used as a carbon source. As shown in figure 1A, the length of the G1 phase also gradually increased from 105 to 150 minutes when the flux of glucose was lowered from 55 fmol $cell^{-1}$ h^{-1} to 14 fmol $cell^{-1}$ h^{-1}. When the glucose flux decreased below 14 fmol $cell^{-1}$ h^{-1}, the G1 phase rapidly elongated to 600 minutes at a glucose flux of 7.5 fmol $cell^{-1}$ h^{-1}. The length of the G1 phase was however shorter when cells were grown on glucose compared to cells grown on galactose with the same flux. The switch point between short and elongated G1 phases was 14 and 18 fmol $cell^{-1}$ h^{-1} for glucose and galactose respectively. The biphasic relation between the length of the G1 phase and the provided carbon flux appeared a general feature.

Accumulation of trehalose and glycogen is responsible for G1 phase elongation

Trehalose and glycogen are accumulated during the G1 phase (6) and the increase in accumulation of trehalose and glycogen correlates with the increase in G1 phase duration in carbon-limited fed-batch cultures (7). To investigate the role of trehalose and glycogen accumulation in G1 phase elongation, we determined the length of the G1 phase in cells that are unable to store both reserve carbohydrates. The effect of trehalose and glycogen accumulation was studied in synchronous fed-batch cultures. Both wild type cells and cells unable to accumulate trehalose and glycogen (SCU10) were synchronized by elutriation in early G1 phase. Both yeast strains were grown in fed-batch cultures at different galactose consumption rates. The wild type cells and the SCU10 cells had a short G1 phase of 105 and 130 minutes respectively at a galactose flux of 55 fmol $cell^{-1}$ h^{-1} (figure 1B). The G1 phase of the wild type cells was elongated to 520 minutes when the galactose flux was decreased to 14 fmol $cell^{-1}$ h^{-1}. The G1 phase of SCU10 cells however was approximately 120 minutes. The SCU10 cells completed their cell cycle, because daughter cells were observed after 200 minutes of culturing. We therefore conclude that cells are able to progress through the entire cell cycle in absence of trehalose and

glycogen accumulation under conditions of low nutrient availability. In addition, we conclude that the accumulation of trehalose and glycogen is responsible for the elongation of the G1 phase in wild type cells under low galactose consumption rates.

G1 cyclin and SBF complex protein expression during a short G1 phase

Earlier work showed the correlation between the length of the G1 phase on one hand and the mRNA expression of the different G1 cyclins (*CLN1*, *CLN2* and *CLN3*) and of the two components of the transcription factor complex SBF (*SWI4* and *SWI6*) on the other (6). To gain more insight in the regulation of the G1 phase, the protein expression patterns of the G1 cyclins Cln1p and Cln3p, and Swi4p and Siw6p were investigated during growth of elutriated cells on glucose or galactose, and on both carbon sources with a relative short and an elongated G1 phase. When the cells were grown with 55 fmol glucose cell^{-1} h^{-1}, the G1 phase was 105 minutes (figure 1A). The protein levels of Cln1 and Cln3 during this short G1 phase are shown in figure 2. At the early G1 phase, Cln1p was present at a low level. The amount of Cln1p increased during the G1 phase and remained at that level after entrance of the

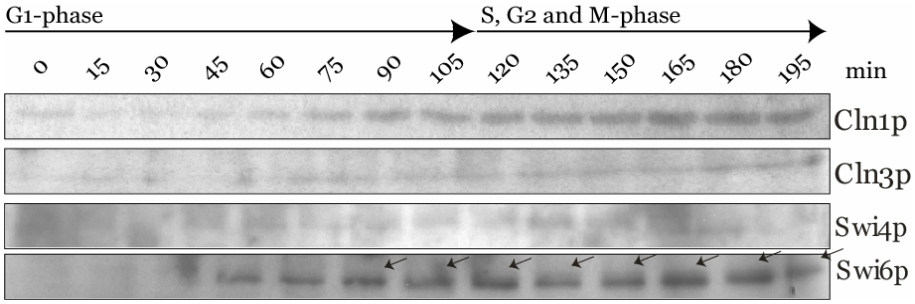

Figure 2. **Cell cycle distribution of Cln1p, Cln3p, Swi4p and Swi6p during G1 phase of 105 minutes on glucose.** Elutriated, early G1 phase cells were grown with a flux of 55 fmol glucose cell^{-1} h^{-1}. Cell lysates were subjected to gel electrophoresis and western blotting as described in materials and methods. Blots were detected with anti-Cln1p, anti-Cln3p, anti-Swi4p or anti-Swi6p specific antibodies. The G1 and S/G2/M phase are indicated at the top of the figure. Arrows point at the phosphorylated form of Swi6p.

S phase. Cln3 protein was present in the early G1 phase cells, and increased during the G1 phase. After entry into the S phase, the amount of Cln3 protein remained constant. The increase of Cln3p expression during the G1 phase was less intense than that of Cln1p during the same period. This indicates that the protein expression of Cln1 is cell cycle elongated and that of Cln3p is not.

Because the transcription factor complex SBF is involved in the regulation of transcription of the G1 phase cyclins, the protein expression patterns of Swi4 and Swi6 were investigated. Both Swi4p and Swi6p appeared during the first half of the G1 phase (figure 2). During the remaining part of the G1 phase, the level of Swi4p and Swi6p increased. The amount of Swi4p seemed to decrease when the culture reached the S phase in contrast to Swi6p levels. A second band that migrated slower on gel was recognized by the anti-Swi6 antibody in cells that entered late G1 phase and S phase (figure 2, arrows). This slower migrating band might represent the phosphorylated form of Swi6p as this was reported to migrate slower on SDS-PAGE gels (32). This suggested Swi6p phosphorylation was consistent with the reported change in localization of Swi6 from the nucleus to the cytoplasm at the end of the G1 phase, which is dependent on the phosphorylation of Swi6 (15, 17).

Elongation of the G1 phase influences the protein expression patterns of Cln1, Cln3, and Swi6

Because the mRNA expression patterns of the G1 phase cyclins and of the SBF components SWI4 and SWI6 were influenced by elongation of the G1 phase (6), the protein expression patterns of Cln1, Cln3, Swi4 and Swi6 were investigated under similar conditions. The increase in G1 phase duration was achieved by a decrease of the glucose flux to 9 fmol $cell^{-1}$ h^{-1}, which resulted in a G1 phase of 420 minutes. Clnp1 was present at the beginning of the G1 phase, and the expression remained constant during the first half of the G1 phase (figure 3). The amount of Cln1 protein decreased during the second half of the G1 phase and after the culture had entered the S phase, Cln1p disappeared completely. Cln3p was also present at the early G1 phase and gradually increased during the remainder of the G1 phase. Cln3 protein levels remained

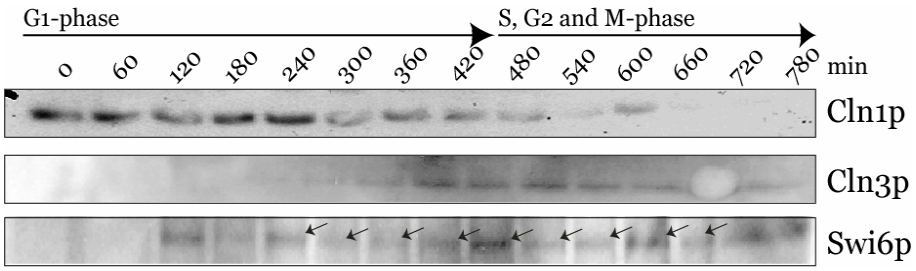

Figure 3. **Cell cycle distribution of Cln1p, Cln3p and Swi6p during a G1 phase of 420 minutes on glucose.** Elutriated, early G1 phase cells were grown with a glucose flux of 9 fmol cell^{-1} h^{-1}. Cell lysates were subjected to gel electrophoresis and western blotting as described in materials and methods. Blots were detected with anti-Cln1p, anti-Cln3p and anti-Swi6p specific antibodies. The G1 and S/G2/M phase are indicated at the top of the figure. Arrows point at the phosphorylated form of Swi6p.

at that level when the culture had entered the S phase after 420 minutes (figure 3). Swi4p was difficult to detect during the cell cycle with a short G1 phase, but it was not at all detectable when the G1 phase was elongated (data not shown). While both Cln1p and Cln3p were detectable in the early G1 phase, Swi6 protein was not present at the early G1 phase but appeared in the first half of the G1 phase and Swi6p persisted during remainder of the G1 phase and after the culture had entered the S phase (figure 3). Before the culture entered the S phase, a second band was also recognized by the anti-Swi6 antibody, probably representing the phosphorylated form of Swi6p (figure 3, arrows). The slower migrating form of Swi6 disappeared between 2.5 and three hours after the culture entered the S phase, while the faster migrating Swi6p band was still present. This pattern corresponds to the reported localization of Swi6 in the cytoplasm during the S, G2 and M phases (15, 17).

Cln1 and Swi6 protein expression patterns during growth on galactose

Because the G1 phase was elongated from 105 to 135 minutes when 55 fmol galactose cell^{-1} h^{-1} was used instead of 55 fmol glucose cell^{-1} h^{-1}, Cln1 and Swi6 protein levels were also determined during growth on galactose with

fluxes of 55 fmol cell^{-1} h^{-1} and 15 fmol cell^{-1} h^{-1} (figure 4A and B, respectively). Culture of synchronous early G1 phase cells with 55 fmol galactose cell^{-1} h^{-1} resulted in a G1 phase of 135 minutes, while the duration of the G1 phase increased to 420 minutes when the cells were cultured with 15 fmol galactose cell^{-1} h^{-1} (figure 1A). Cln1 protein was already present at the early G1 phase in the G1 phase of 135 minutes (figure 4A) and increased during the remainder of the G1 phase. After the cells had entered S phase, the Cln1p signal remained constant and this expression pattern was comparable to the pattern after growth of elutriated cells on glucose with a G1 phase of 105 minutes. Swi6 protein appeared during the first half of the G1 phase and decreased when the culture had entered S phase (figure 4A). A band of slower migrating Swi6p appeared at the end of the G1 phase, somewhat earlier in the G1 phase as during growth on glucose with a G1 phase of 105 minutes (figure 4A, arrows), although the absolute time of appearance was the same. Slower migrating Swi6p disappeared after the cells had entered the S phase, but the faster

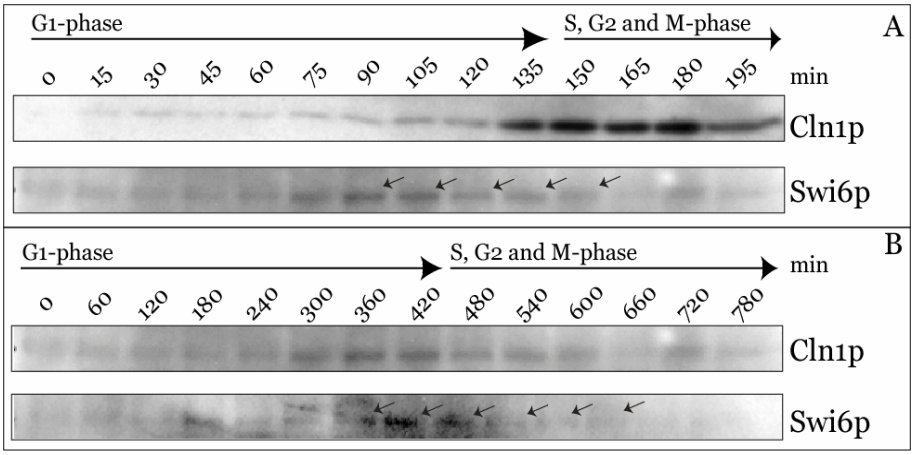

Figure 4. **Cell cycle distribution of Cln1p and Swi6p during G1 phases of 105 minutes (A) and 420 minutes (B) on galactose.** Synchronized, early G1 phase cells were grown with a galactose flux of 55 fmol cell^{-1} h^{-1} (panel A) or 15 fmol cell^{-1} h^{-1} (panel B). Cell lysates were subjected to gel electrophoresis and western blotting as described in materials and methods. Blots were detected with anti-Cln1p, and anti-Swi6p specific antibodies. The G1 and S/G2/M phase are marked at the top of each panel. Arrows point at the phosphorylated form of Swi6p.

migrating Swi6p signal remained present.

Lowering the galactose flux to 15 fmol $cell^{-1}$ h^{-1} resulted in a G1 phase of 420 minutes (figure 1A). Cln1p was present at a low level in the early G1 phase cells (figure 4B). The amount of Cln1 increased during the first half of the G1 phase and decreased in late G1 phase, and finally disappeared completely upon entrance of S phase. This pattern was different from the Cln1 protein pattern during growth on glucose with a G1 phase of 420 minutes when Cln1p was most prominent present during the first half of the G1 phase. Swi6 protein appeared during the first half of the G1 phase, which was similar to levels measured in glucose and a high galactose flux cultures. Swi6 protein levels increased during the remaining G1 phase. The cells entered S phase after 420 minutes and the amount of Swi6p decreased and disappeared completely (figure 4B). Slower migrating Swi6p appeared at the end of the G1 phase and this was comparable to the appearance of slower migrating Swi6p during growth on glucose or at a high galactose flux. This second Swi6p band increased together with the signal of faster migrating Swi6p during late G1 phase and was gone when the faster migrating band disappeared after the culture had entered S phase. The Swi6 protein patterns corresponded to the protein pattern during growth on glucose with a G1 phase of 420 minutes. Therefore, we conclude that the pattern of presence of the slower migrating Swi6 is dependent on the length of the G1 phase.

Taken together, these data indicate that progression through the G1 phase is similar for the carbon sources and G1 phase lengths investigated in this study, although some differences result from growth on either galactose or glucose. These differences might result from the lower energy yield of galactose catabolism compared to glucose catabolism.

SLT2 is required for the presence of slower migrating Swi6p

In response to heat stress, Swip6p is phosphorylated by active Slt2p in wild type cells and no phosphorylated Swi6p was detected in *slt2*Δ cells (32). This phosphorylation of Swi6p halts the cell cycle in the G1 phase because the

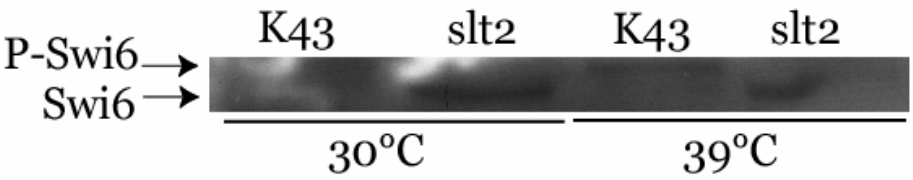

Figure 5. **Swi6p is not phosphorylated in *slt2*Δ cells.** Wild type (K43) and *slt2*Δ (slt2) cells were grown to exponential phase at 30°C on medium supplemented with 1 M sorbitol. Half of the culture of each strain was heat shocked for 60 minutes at 39°C. Cells were collected and lysates were subjected to western blotting as described in materials and methods.

transcription of G1 phase specific genes is stopped. This also enables Swi4p alone to induce transcription of cell wall genes in order to withstand the stress.

In order to confirm that the slower migrating Swi6p band observed by Western blotting in our experiment represents the phosphorylated form of Swi6p, we determined whether this band is present in *slt2*Δ cells. Wild type cells and *slt2*Δ cells were cultured to the exponential phase. Half of the culture was heat shocked for one hour at 39°C. Samples were collected and the cells were lysed. Lysates were subjected to western blotting and Swi6 proteins were detected. In wild type cells, a fraction of Swi6p migrated slower at 30°C and this fraction increased upon heat shock (figure 5). In the *slt2*Δ cells, only the faster migrated band of Swi6p was detected at both 30°C and 39°C. Therefore, we conclude that the slower migrating band recognized by the anti-Swi6p antibody represents the phosphorylated form of Swi6p.

The phosphorylation of Slt2 during the cell cycle is correlated to the presence of slower migrating Swi6p

To investigate whether the cell cycle-dependent Swi6p phosphorylation correlated with cell cycle-dependent activity of Slt2p, phosphorylated Sltp2 was detected during the cell cycle. The phosphorylation of Slt2 is coupled to its activity and active Slt2p is therefore determined by antibody detection of phosphorylated Slt2p (51). In early G1 phase cells, Slt2 was not phosphorylated and no phosphorylated Slt2p was detected before the mid of the G1 phase when elutriated cells were grown to a G1 phase of either 105 or 420 minutes (figure 6). During growth with a G1 phase of 105 minutes,

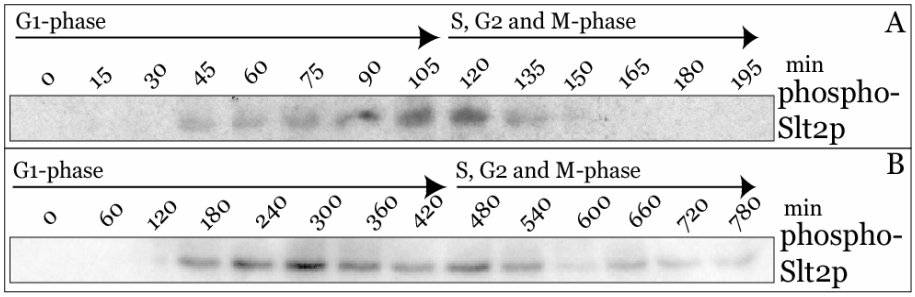

Figure 6. **Cell cycle distribution of active Slt2p (pSlt2p).** Elutriated cells were grown with a glucose flux of 55 fmol cell^{-1} h^{-1} (panel A) or 11 fmol cell^{-1} h^{-1} (panel B). Cell lysates were subjected to gel electrophoresis and western blotting. Blots were detected with anti-phosphorylated (active) Sltp2 specific antibodies. The G1 and S/G2/M phase are stated at the top of each panel.

the Slt2p phosphorylation was high during late G1 and early S phase. The phosphorylation of Slt2p slowly decreased and disappeared after S phase entry. The appearance of phosphorylated Slt2p preceded the band of phosphorylated Swi6p, while this Swi6p band persisted after the disappearance of phosphorylated Slt2p.

During growth with a G1 phase of 420 minutes, Slt2p became also phosphorylated at mid-G1 and the maximum signal was achieved during the second half of the G1 phase. At the end of the G1 phase, the phosphorylation of Slt2p decreased but remained present during the rest of the cell cycle. This phosphorylation pattern corresponded with the presence of phosphorylated Swi6.p Our data suggest that there might be a coupling between Swi6p phosphorylation and Slt2p activity during the cell cycle, because the phosphorylation of Swi6p is dependent on the activity of Slt2p (32) and phosphorylated Slt2p preceded the presence of the phosphorylated Swi6p band.

Deletion of SLT2 results in severe cell cycle delay

Because Slt2p phosphorylation seemed to be cell cycle regulated and seemed to be involved in the regulation of Swi6p during the G1 phase, we investigated whether *SLT2* was required for proper cell cycle progression. The use of chemicals such as PD098059 to inhibit Slt2p activity in wild type cells was not possible because these chemicals do not pass the yeast cell wall.

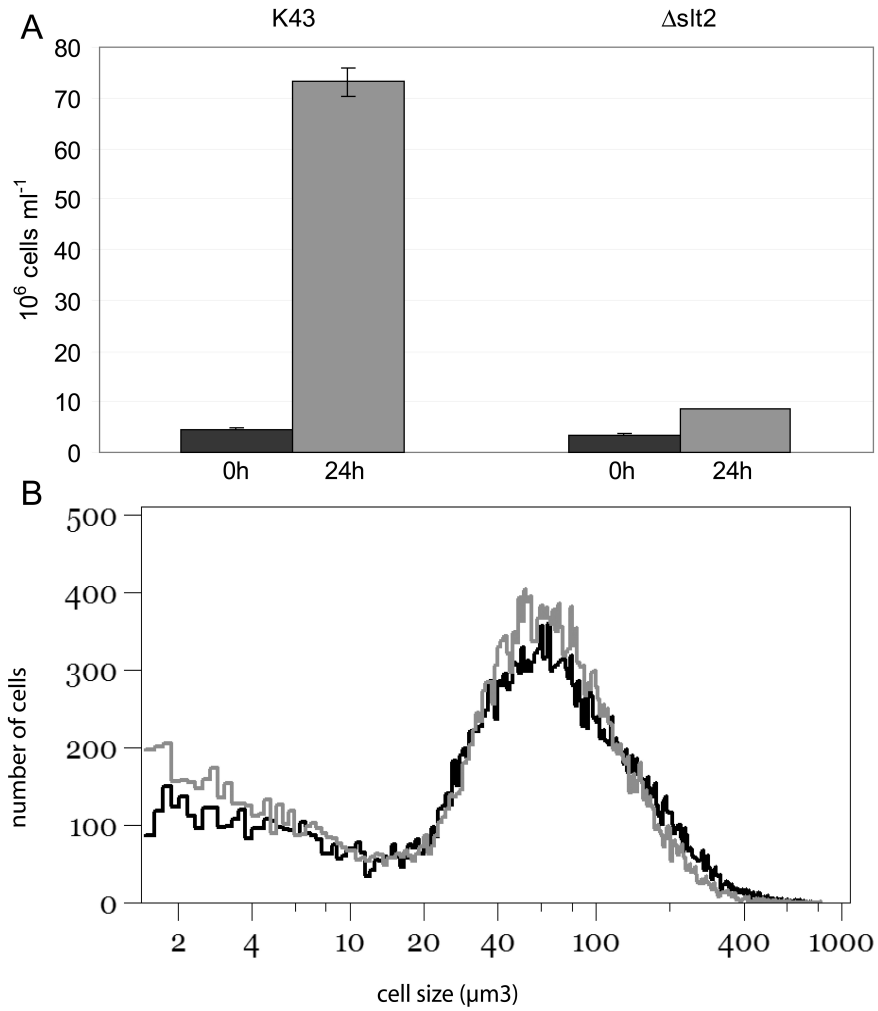

Figure 7. **Cell cycle requirement of SLT2.** Increase in cell concentration of wild type (K43) and *SLT2* deletion strain (Δslt2) from the beginning (0h) to after 24 hours (24h) of batch growth on medium supplemented with 1M sorbitol (A). Cell size measurement on Coulter MultiSizer II shows no massive cell lysis after 24 hours of batch culturing for wild type cells (red) or *slt2*Δ cells (green) (B). Sample amount was selected to give comparable amplitudes in the graphs. The cell concentrations were subsequently calculated by Coulter® Multisizer AccuComp®.

Therefore, a *slt2*Δ mutant was used for this experiment. Both the mutant strain and the wild type strain were cultured in medium supplemented with sorbitol, which prevented cell lysis of the deletion mutant. Both strains were inoculated at a concentration of $4*10^6$ cells/ml and grown in batch culture for 24 hours. In

figure 6 is shown that the wild type culture after 24 hours had a concentration of $73*10^6$ cells/ml and thus produced $69*10^6$ daughter cells/ml (figure 7A). The *slt2*Δ mutants strain had after 24 hours of batch growth only $8*10^6$ cell/ml and thus produced only $4*10^6$ daughter cells/ml. Because no lysed cells were observed under the microscope and cell size analysis lacked a peak in small particles (figure 7B), no massive cell lysis had appeared in the wild type or the *slt2*Δ strain. The lower amount of daughter cells after 24 hours in the *slt2*Δ strain was therefore not the result of massive cell lysis. After multiple (4 to 5) days of growth, the *slt2*Δ strain was also able to reach a similar cell concentration as the wild type strain and therefore we conclude that deletion of *SLT2* caused large cell cycle elongation and that *SLT2* is required for proper cell cycle progression.

In summary, our data show that the amount of carbon source provided to early G1 phase cells determines the length of the G1 phase and thereby the protein expression patterns observed. The second band of Swi6 protein observed is dependent on the activity of Slt2p and was proven to be the phosphorylated form of Swi6p. The activity of Slt2p is cell cycle regulated and deletion of this kinase leads to a severe cell cycle progression delay.

Discussion

In this study, we investigated the progression though the G1 phase of the cell cycle with synchronized, early G1 phase cells. With this method we obtained synchronous cells without interfering the cell cycle with chemicals or mutants (45-47). The use of a fed-batch system with different galactose fluxes allowed us to regulate the length of the G1 phase (6, 7) and we show here that the biphasic relationship between the length of the G1 phase and the carbon flux provided to the cells is also applicable to fed-batch culturing with different glucose fluxes. The switch point between short and elongated G1 phases was 18 fmol $cell^{-1}$ h^{-1} for galactose (7), and 14 fmol $cell^{-1}$ h^{-1} for glucose, reflecting the higher energy yield of glucose compared to galactose. The G1 phase is not elongated in cells deleted for the ability to store trehalose and glycogen. The accumulation of trehalose and glycogen is thus responsible to elongate the G1

phase. Under G1 phase elongation conditions, the energy stored in reserve carbohydrates is not available for cell cycle progression, which is slowed down as a result.

The progression through the G1 phase was monitored by measurements of the budding percentage. The protein expression pattern of G1 cyclins Cln1 and Cln3 demonstrated differences depending on the length of the G1 phase. Culturing of synchronized, early G1 phase cells with 55 fmol glucose or galactose resulted in different G1 phase lengths. This probably explains the differences in protein expression patterns of Cln1 on glucose and galactose. The expression of Cln3p was not altered by changes in length of the G1 phase. The gradual increase in Cln3 protein expression correlated with the mRNA expression data obtained by Silljé et al (6), but the pattern of Cln1 protein was not comparable to the mRNA data. The discrepancy between RNA and protein expression patterns of *CLN1* might result from residual Cln1p from the previous cell cycle, or by more sensitive detection of Cln1p with antibodies than the detection of *CLN1* by Northern blot analysis.

The protein expression of Swi4 appeared to be higher during a G1 phase of 105 minutes than during a G1 phase of 420 minutes. The decreased synchrony during the G1 phase of 420 might account for lowering of the Swi4p signal. *SWI4* mRNA/Swi4p is not continuously expressed during the cell cycle and the amount of cells that express Swi4p at the times of sampling is therefore decreased (6). The lower synchrony during elongated G1 phase might decrease in this way the signal of the culture below the detection limit while the total amount of Swi4p expression per cell during the period of Swi4p expression is not altered. Swi6p was detectable during both G1 phase lengths. The Swi6p expression was different for glucose and galactose during a G1 phase of 105 minutes. The Swi6p expression was similar on glucose and galactose culturing with G1 phases of 420 minutes. The slower migrating Swi6 band represented the phosphorylated form of Swi6p that is known to appear at the end of the G1 phase and persist throughout the S, G2 and M phase and only disappears when the cells exit mitosis (15, 17). This Swi6p phosphorylation is required for exit of Swi6p out of the nucleus at the end of the G1 phase and its localization in the cytoplasm during the other phases of the cell cycle, and via

this localization for proper cell cycle progression. The presence of the slower migrating band of Swi6p corresponded with the known translocation of Swi6p from the nucleus to the cytoplasm.

The phosphorylation and the localization of Swi6p is dependent on the activity of the MAP kinase Slt2p (15, 17, 32). Slt2p is the executing kinase of the Pkc1 cell wall integrity pathway which is involved in maintenance of the cell wall (29). The activation of the Pkc1 pathway is known to be partially dependent on the Cdc28p, but it is unknown which cyclin is involved in this process (23). In this study we show that Slt2p is activated in mid-G1 phase during growth on glucose for both short and elongated G1 phases. This activation preceded the appearance of the phosphorylated Swi6p band in both G1 phase lengths. The G1 cyclin involved in activation of Slt2p could not be denoted from the results of our experiments because both Cln1p and Cln3p proteins were already present before phosphorylated Slt2p appeared. The activity of Slt2p was shown to be critical for proper cell cycle progression, as deletion of *SLT2* resulted in severe cell cycle elongation. The percentage of budded cells increased in the *SLT2* deletion mutant compared to the percentage of budded wild type cells during the exponential phase in asynchronous batch growth (data not shown). This indicates that the *slt2*Δ cells arrest at the second cell cycle restriction point, in the G2 phase, called the cell morphology checkpoint.

The MAPK cascades are preserved from yeast to mammals (34, 35). In mammalian cells, inhibition of the p42/p44 MAPK by the use of antisense constructs, overexpresssion of kinase inactive mutants or inactivation by MPK-1 blocks DNA synthesis and cell proliferation (41, 42).However, inhibition of MAPK by the use of MEK inhibitor PD-98059 severely inhibited DNA synthesis but did not block DNA synthesis completely (40). High-level activity of p42/p44 MAPK results in cell cycle arrest (52, 53). In *S. cerevisiae*, inhibition of Slt2 activity also results in severe cell cycle elongation. Although no data are published about the direct link between increased Slt2 activity and cell cycle progression, elevated Slt2 activity after stress is known to correlate with cell cycle delay. *E.g*. mild heat shock and osmotic shock are known to delay both bud formation and nuclear division and both stresses are

demonstrated to increase Slt2 activity (54-57). Thus, the regulation of cell cycle progression might be conserved from yeast to mammals for the role of MAPK. Not only is the activity of MAPK required for cell cycle progression in both yeast and mammals, overactivity of MAPK is correlated with cell cycle delay.

On the other hand, the activation of Slt2p is considered to be a side-effect of trehalose accumulation under stress conditions. Heat shock results in accumulation of trehalose, which increases the internal osmolarity of the cell. The activation of Slt2 can under these conditions be prevented by osmotic stabilization of the medium and the activity of Slt2p is also decreased in mutants unable to accumulate trehalose in which the internal pressure is not elevated during heat stress (58). It is however not known whether the cell cycle is elongated in these mutants or under the osmotic stabilizing conditions. More research is therefore necessary to investigate the possibility of secondary Slt2p activation under stress conditions. The activity of Slt2p is required for normal cell cycle progression, but the prolonged activity under nutrient limitation might thus be caused by the accumulation of trehalose.

In general, the present study shows the correlation between G1 phase length and the expression of Cln1p, Cln3p and Swi6p, and of Slt2p phosphorylation. The expression of the different proteins is positively regulated by the availability of nutrients, while the phosphorylation of Slt2p is increased upon nutrient deprivation (59-63). The expression of these proteins in turn determines the length of the G1 phase and this report demonstrates the expression patterns of these proteins during short and elongated G1 phases. The phosphorylation of Slt2p is regulated by the length of the G1 phase and is required for proper cell cycle progression.

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Chapter 4

Involvement of *HXT5* in trehalose accumulation and in glucose transport after gluconeogenesis in *Saccharomyces cerevisiae*

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Abstract

Hexose transporter (Hxt) proteins facilitate the uptake of glucose in *Saccharomyces cerevisiae*. The expression of the major hexose transporters (*HXT1-4* and *HXT6/7*) is regulated by the extracellular glucose concentration, while the expression of *HXT5* is regulated by the growth rate. The accumulation of trehalose is important for stress-resistance and this accumulation is regulated by the growth rate of the cells. This study shows that especially the expression of *HXT5* correlated with the accumulation of trehalose during batch growth, fed-batch culturing and in continuous cultures, but none of the hexose transporters was absolutely required for trehalose accumulation. In addition, Hxt5p coimmunoprecipitated with Tps1p after addition of glucose to starved cells, suggestion that Hxt5p forms a functional complex with Tps1. Surprisingly, Hxt5p was mainly localized to the endoplasmic reticulum during the stationary phase and only a small fraction was localized to the plasma membrane. Homology with a glucose transporter in the endoplasmic reticulum of mammalian cells suggests a role for *HXT5* in gluconeogenesis.

Introduction

Hexose transporter (Hxt) proteins facilitate the uptake of glucose in *Saccharomyces cerevisiae*. The hexose transporter family consists of 20 members, of which *HXT1-4* and *HXT6/7* are the major hexose transporters. The expression of the major hexose transporters is regulated by the extracellular glucose concentration (2-9). In a strain deleted for *HXT1-7*, growth on glucose requires at least one of the major hexose transporters (10). Besides the major hexose transporters, only expression of *HXT5* under its own promoter enables growth on glucose, although only at low growth rate (11). Therefore, *HXT5* was initially not identified as capable of restoring growth on glucose when *HXT5* was the only hexose transporter expressed in a *HXT1-7* null mutant (11).Overexpression of *HXT8-11* or *HXT13-17* restores growth on glucose as well in a strain deleted for *HXT1-17* and *GAL2* (12).

HXT1 expression is induced when the glucose concentration in the medium is high and is characterized as a low-affinity hexose transporter (3, 9). Both *HXT2* and *HXT4* become expressed when the glucose concentration is low and are considered to be high-affinity hexose transporters (3, 13). The expression of *HXT3* is not dependent on the concentration, but only on the presence of glucose and Hxt3p is defined as a hexose transporter with intermediate affinity (3, 4, 6). *HXT6* and *HXT7* are induced under low glucose conditions, and are not expressed after glucose depletion. Although *HXT6* and *HXT7* only differ in two amino acids within the encoded proteins, *HXT7* expression levels are higher than those of *HXT6* (2, 7).

The expression of *HXT5* is not regulated by the extracellular glucose concentration, but by the growth rate of the cells (14). Studies of the promoter of *HXT5* revealed two stress-responsive elements (STREs), two HAP2/3/4/5p binding sites (HAP elements) and one post-diauxic shift (PDS) element (15). STRE elements are involved in stress responses (16) and one of the STRE elements in the *HXT5* promoter was shown to be required for *HXT5* expression (15). HAP elements are implicated to positively influence transcription during growth on non-fermentable carbon sources (17-20) and the HAP elements in the *HXT5* promoter indeed seem to be involved in transcription after glucose depletion on batch growth or during growth on non-fermentable carbon sources (15). PDS elements are involved in gene expression during the diauxic shift and are regulated by the Ras/cAMP pathway (21). The PDS element in the *HXT5* promoter is suggested to regulate expression during growth on ethanol (15).

Glucose that is transported into the cell is immediately phosphorylated by a hexokinase (Hxk) to give glucose-6-phosphate (22). Dependent on the conditions of the cells, glucose-6-phosphate enters glycolysis or is used to synthesize the reserve carbohydrates trehalose and glycogen (23-25). Typically, the growth rate of the cells determines whether reserve carbohydrates are stored or not, and a lower growth rate results in higher accumulation of trehalose and glycogen (1, 26, 27).

Both reserve carbohydrates are involved in cell survival; deletion of the ability to store one of them leads to a minor decrease in cell survival, but the

inability to store both drastically lowers the percentage of viable cells after an incubation of several days (27, 28). The accumulation of glycogen can be abolished by deletion of *GSY1* together with *GSY2*, while trehalose is no longer stored upon deletion of *TPS1* (29-32). The other two members of the trehalose accumulation (TPS) complex, *TPS3* and *TSL1*, appear to serve as regulators and are not required for trehalose accumulation (33). Deletion of the ability to store trehalose by knocking out *TPS1* caused an immediate arrest of growth of these cells on glucose or fructose, irrespective on the ability to accumulate glycogen (34, 35). It has been suggested that Tps1p itself, or the product trehalose-6-phosphate, inhibits the import of glucose into the cell by regulating Hxt proteins or the influx of glucose into the glycolysis by modulation of the hexokinases (29, 33, 36). The importance of Tps1action is reflected by the increase in early glycolysis intermediates and the depletion of ATP when glucose is added to *tps1*Δ cells. This overflow of the glycolysis and depletion of ATP renders it impossible for the cell to generate energy (ATP) and the cells become literally energy exhausted (34-38).

Considering the proposed interaction of Tps1p with one or more hexose transporters (36) and the growth defect of a *tps1*Δ strain on glucose (34-38), we investigated the specific involvement of the hexose transporters in trehalose accumulation. Because the precursor of trehalose is glucose, the major hexose transporters were included in this study together with *HXT5* which is reported to be expressed under trehalose synthesis conditions. The data presented in this study show that none of the major hexose transporters or *HXT5* was specifically involved in the accumulation of trehalose. *HXT5* was the only hexose transporter always expressed under trehalose accumulating conditions during batch and fed-batch culturing and in continuous cultures. Hxt5p coimmunoprecipitated with Tps1after glucose addition to stationary phase cells. This suggested a role of the Hxt5-Tps1 interaction after the addition of glucose to starved cells. In this context, the existence of a glucose-response complex (GRC) is suggested and discussed.

Surprisingly, electron microscopy on cryosections labelled for Hxt5-HA revealed that Hxt5-HA was localized mainly to the endoplasmic reticulum during the stationary phase and only a small fraction was localized to the

plasma membrane. In mammalian cells, a glucose transporter is also localized to the endoplasmic reticulum and this transporter is involved in gluconeogenesis. A possible role for Hxt5p in gluconeogenesis is therefore addressed in the discussion.

Materials and Methods

Strains, media and growth conditions

The *S. cerevisiae* strains used in this study are indicated in Table 1. Cells were grown at 30°C at 180 rpm in a shaking incubator (New Brunswick Scientific, Edison, NJ, USA) in 0.67% (w/v) yeast nitrogen base without amino acids (Difco, Sparks, MD, USA) and 2% (w/v) of the carbon source as indicated in the text.

Continuous culture experiments were performed as described previously (14, 39). Cultures were started in EGLI medium as described by Meijer et al., (39) and grown over night. Continuous culturing was then started. The NH_4^+ concentration was adapted to 1.5 $g.l^{-1}$ and the glucose concentration in the feed was 200mM to ensure nitrogen limitation. Samples of steady state situations were taken as described (39).

Fed batch experiments using galactose as carbon source were performed as described (1, 14). In short, cells were grown in YNB medium without amino acids and glucose as carbon source. The initial cell concentration was $1.2*10^7$ $cells.ml^{-1}$, and the glucose concentration in the medium was initially 0.05 mM. Glucose dissolved in YNB medium was administered continuously to the cells at rates of 10 fmol and 50 fmol $cell^{-1}$ h^{-1} respectively. Growth of the culture was monitored by measuring of the OD_{600} at intervals during the experiments.

Northern blot analysis

Total RNA was isolated, separated on agarose gels and transferred to Hybond-N membrane (Amersham Pharmacia Biotech, Buckinghamshire, United Kingdom) as described earlier (14). Oligonucleotides directed against *HXT1-7* (4) were labelled as described (14). A probe against *U2* was obtained

by PCR, using plasmid pRS313U2 as template and primers 5'-GTTTTGCAAGGAAAGG-3' and 5'-CGAGAGAAACATCAAGCG-3'. The probe was labelled with [α-^{32}P]dCTP using prime-a-gene labelling system according to manufacturer's protocol (Promega, Madison, Wisconsin, USA). Hybridization and washing was performed as described earlier (14).

Table 1: List of strains used:

Yeast strain	Genotype	Source
CEN.PK 113-7D (wild type)	MAT**a** *SUC2 MAL2-8^c*	P. Kötter (Frankfurt, Germany)
CEN.PK 113-3C Δtps1	MAT**a** *SUC2 MAL2-8^c tps1::TRP1*	(28)
CEN.PK 441-3B Δhxt1	MAT**a** *SUC2 leu2-3,112, hxt1::loxP*	P. Kötter (Frankfurt, Germany)
CEN.PK 441-3B Δhxt2	MAT**a** *SUC2 leu2-3,112, hxt2::loxP*	P. Kötter (Frankfurt, Germany)
CEN.PK 441-3B Δhxt3	MAT**a** *SUC2 leu2-3,112, hxt3::loxP*	P. Kötter (Frankfurt, Germany)
CEN.PK 441-3B Δhxt4	MAT**a** *SUC2 leu2-3,112, hxt4::loxP*	P. Kötter (Frankfurt, Germany)
MSY1 (Δhxt5)	MAT**a** *MAL2-8^c SUC2 leu2-3,112 Δhxt5::LEU2*	(11)
CEN.PK 441-3B Δhxt6/7	MAT**a** *SUC2 leu2-3,112, hxt6/7::loxP*	P. Kötter (Frankfurt, Germany)
KY98 (Hxt5-GFP)	MAT**a**, *SUC2, MAL2-8^c , HXT5::GFP*	A. Kruckeberg (Amsterdam, the Netherlands)
JBY20 (Hxt5-HA)	MAT**a**, *SUC2*, *MAL2-8^c*, *ura3*, *HXT5::HA*	Jessica Becker (Düsseldorf, Germany)

Determination of trehalose and glucose levels

The amount of accumulated trehalose and the concentration of glucose in the medium were determined enzymatically as described previously (1).

Western blot analysis

Equal amounts of cells were collected by centrifugation, washed with ice-cold water and stored at -20°C until use. RIPA buffer (150 mM NaCl, 20 mM Tris-HCl pH 7.5, 1 mM EDTA, 1% SDS and 0.1% Triton X-100) complemented with protease inhibitors (Complete, Roche Diagnostics, Basel, Switzerland) was used as lysis buffer and cells were lysed by vigorously shaking with 0.45 mm glass beads in a Mini BeadBeater 8 (Glen Mills Inc., New Jersey, USA). After SDS-PAGE, the proteins were transferred to PVDF membranes (Roche Diagnostics). For detection, the membranes were blocked in PBS pH 7.4 (137 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4), 0.1% Tween-20, 3.4% Protifar Plus (Nutricia, the Netherlands) and subsequently incubated with anti-Hxt1, anti-Hxt3, anti-Tps1 rabbit polyclonal or anti-HA (12CA, Roche Diagnostics) mouse monoclonal antibodies. The antibodies were used at a dilution of 1:500, diluted in PBS, 0.1% Tween-20, 3.4% protifar plus. After three washes of ten minutes each with PBS, 0.1% Tween-20, 1% protifar plus, the primary antibody was detected with peroxidase-conjugated goat-anti-rabbit or rabbit –anti-mouse (Jackson Immunoresearch Antibodies Laboratories, Inc., Westgrove, PA, USA)) at a dilution of 1:10,000 in TBS, 0.1% Tween-20 or PBS, 0.1% Tween-20. After three washes of ten minutes each with PBS, 0.1% Tween-20, 1% protifar plus, the proteins were visualized with Enhanced Chemiluminescence (Renaissance, NEN Life Science Products, Inc., Boston, MA, USA).

Immunoprecipitation

Equal amounts of Hxt5-GFP expressing cells were washed once with ice-cold H_2O and cells were collected by centrifugation. Cells were suspended in 500 µl PBS supplemented with complete protease inhibitors (Roche) and 20 mM NaF, and subsequently the cells were lysed by vigorously shaking with

0.45 mm glass beads in a Mini BeadBeater 8for 1.5 min. 500 µl PBS containing protease inhibitors, 20 mM NaF and 1% Tween-20 (Sigma Chemical Company, St. Louis, MO, USA) was added, the sample was mixed and 800 µl lysate was collected. Tps1 was immunoprecipitated from pre-cleared lysates with the use of anti-Tps1 rabbit polyclonal antibody coupled to protein G sepharose beads (Sigma Chemical Company). After binding, the beads were washed four times with PBS containing protease inhibitors, 20 mM NaF and 0.5% Tween-20. 1.2x reducing sample buffer was added and immunoprecipitates were separated on SDS-PAGE gel. The proteins were transferred to PVDF membrane (Roche Diagnostics) and detected with anti-GFP mouse monoclonal antibody (Roche Diagnostics) at a dilution of 1:500 and rabbit-anti-mouse peroxidase (Jackson Immunoresearch) at a dilution of 1:10,000, and visualized with Enhanced Chemiluminescence (Renaissance). Blocking, washing and buffers are described above under Western blotting.

Electron microscopy on cryosections

Cells were fixed for 15 min at 30°C and 180 rpm by adding an equal volume of two times concentrated fixative (4% formaldehyde, 0.4% glutaraldehyde in 0.1 M PHEM buffer (60 mM Pipes, 25 mM Hepes, 10 mM EGTA and 2 mM $MgCl_2$), pH 6.9). Cells were collected by centrifugation for 5 minutes at 4000 rpm and fixation was continued for 45 min at room temperature with fresh fixative (1% formaldehyde, 0.1% glutaraldehyde in 0.1 M PHEM buffer). Then the cells were washed with 0.1 M PHEM buffer and resuspended in 1% formaldehyde in 0.1 M PHEM buffer, and stored at 4°C until further use. The cells were washed three times with 0.1 M PHEM buffer and incubated for one hour at room temperature in 1% $NaIO_4$ in 0.1 M PHEM buffer,. Subsequently, the cells were washed three times with 0.1 M PHEM buffer and embedded in 12% gelatin in 0.1 M PHEM buffer at 37°C. Cells were centrifuged for two minutes at 15000 rpm and gelatin was solidified on ice. 1 mm^3 cubes were cut, which were impregnated overnight on a turning wheel with 2.3 M sucrose at 4°C (40). With a cryoultramicrotome (UC6 FC6, Leica, Microsystems,, Vienna, Austria), 50-55 nm sections were cut for electron

microscopy. Sections were picked up with 1.15 M sucrose/0.1% methylcellulose in 0.05M PHEM buffer, pH 6.9 and dipped on formvar/carbon coated Cu grids (Stork Veco B.V., Eerbeek, the Netherlands) for electron microscopy.

Mounted sections of JBY20 and K43 cells were washed five times with PBS, pH 7.4 (137 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4)/ 0.02 M Glycine. The sections were blocked by one incubation in PBS, 1% BSA (Sigma) and subsequently incubated with mouse 12CA5 (Roche Diagnostics) diluted 1:30 in PBS, 1% BSA for one hour. The labeled sections were washed five times two minutes with PBS, 0.1% BSA and incubated for 20 minutes with rabbit-anti-mouse IgG (Dako Denmark A/S, Glostrup, Denmark,) diluted 1:250 in PBS, 1% BSA. The sections were washed five times two minutes with PBS, 0.1% BSA and then incubated 20 minutes with protein A 10 nm gold (Dept. Cell Biology, University Medical Centre Utrecht, the Netherlands) diluted 1:90 in PBS, 1% BSA. The sections were then rinsed three times shortly and seven times for two minutes with PBS. The labeled sections were fixed for five minutes in PBS, 1% glutaraldehyde and subsequently washed ten times with aqua dest. This was followed by an incubation of five minutes in uranyl oxalate, pH 7.4 (41) and two washes with aqua dest. Two rinses on ice on uranylacetate, methylcellulose, pH 4.5 followed and the incubation was continued with fresh solution for 5 minutes on ice. Excess of fluid was then drained off, and the sections were dried at room temperature.

The sections were observed with a JEOL 1010 EX transmission electron microscope (JEOL, Tachikawa, Japan) at 80 kV and images were recorded on Agfa Scientia sheet film. The scanned pictures were processed with Photoshop 5.5.

Results

Expression of HXT1-5, HXT7 and TPS1 during batch culture

Recently, we have suggested that Hxt5p was a good candidate for regulation of Tps1p activity and consequently of trehalose synthesis (14). To investigate a possible relationship between expression of *HXT5* or one of the major hexose transporters, and the expression of *TPS1* or trehalose

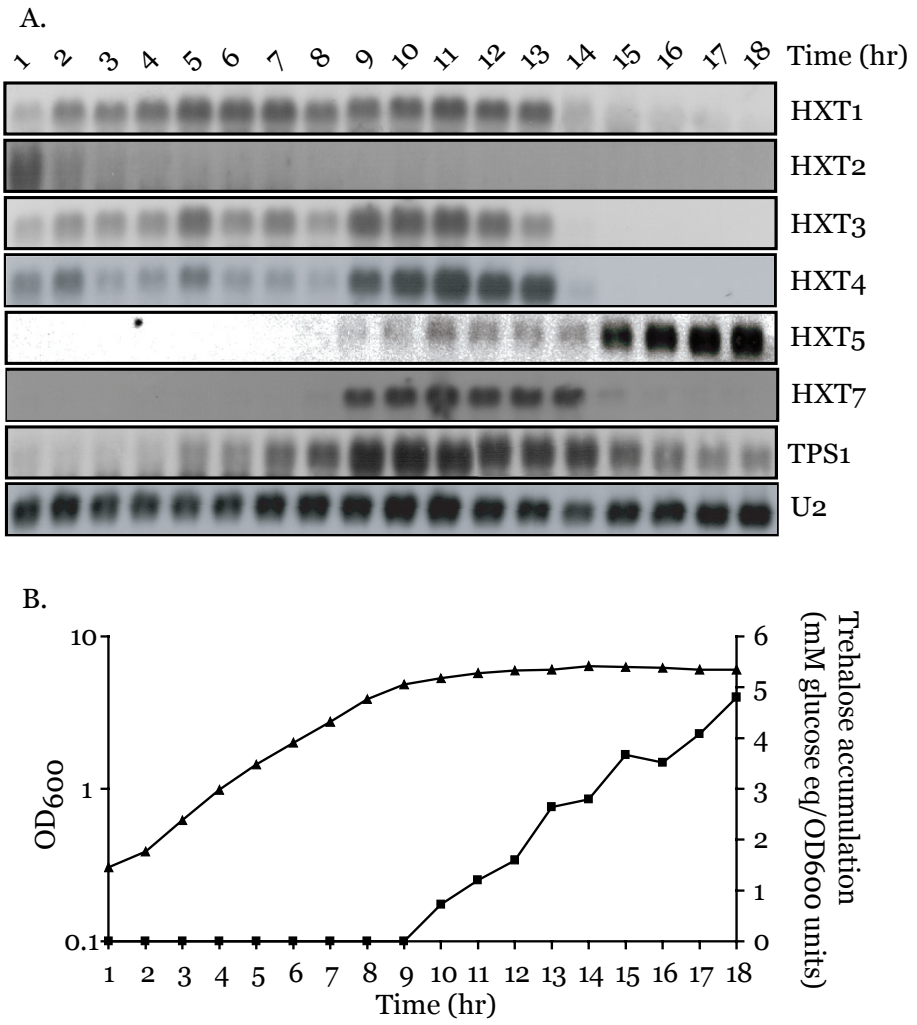

Figure 1. **Trehalose accumulation during batch growth correlates with *HXT5* and *HXT7* expression and is continued in the stationary phase.** Batch growth was inoculated at t=0h on YNB medium containing 2% glucose. Cells were collected each hour and Northern blot analysis of expression of *HXT1-5*, *HXT7* and *TPS1* is presented in panel A. The growth of the culture was monitored by measurement of OD_{600} (B, ▲). Trehalose accumulation was determined enzymatically as described by Silljé et al., (1) (B, ■).

accumulation, we determined the expression of *HXT1-7* and *TPS1*, and the accumulation of trehalose during batch growth on glucose at an initial concentration of 100 mM (figure 1A). *HXT1*, *HXT3* and *HXT4* were expressed from 1 to 13 hours after start of the batch growth. When the glucose

concentration in the medium had declined below 10 mM, expression of *HXT1*, *HXT3* and *HXT4* was repressed. *HXT2* was only expressed at 1 hour after the start of batch growth when the extracellular glucose concentration was still above 100 mM. *HXT5* expression was induced from 9 hours after the beginning of batch growth at an extracellular glucose concentration of 35 mM and was further induced after 13 hours after the initiation of batch growth. This additional *HXT5* expression coincided with exhaustion of glucose in the medium at 15 hours after the start of the batch growth. The expression of *HXT5* was previously shown to depend on the growth rate of the cells (14). At the beginning of *HXT5* expression, the growth rate of the cells had decreased as the culture entered the stationary phase (Fig. 1B). *HXT6* mRNA was not detectable in this experiment (data not shown). *HXT7* was expressed between 9 and 14 hours after the start of batch growth, which is during the growth phase with low glucose concentrations in the medium (decreasing from 47 mM at 9 hours to 4 mM at 14 hours). *TPS1* was already expressed at a low level at the beginning of the experiment; the expression of *TPS1* increased 5 hours after the start of batch growth and was further increased from 9 to 14 hours after the initiation of batch growth. *TPS1* expression was reduced at later time points but remained present at similar levels as compared to 5 to 8 hours after the start of batch growth. Trehalose was however only accumulated after 10 hours after the initiation of batch growth, and this accumulation continued when glucose in the medium was exhausted (figure 1B). During the accumulation of trehalose, *HXT1*, *HXT3*, *HXT4*, *HXT5* and *HXT7* were expressed until the extracellular glucose concentration had declined below 10 mM at 13 hours after the start of batch growth. When the glucose concentration in the medium was still 4 mM (at 14 hours after the onset of batch growth), both *HXT5* and *HXT7* were expressed. After glucose depletion, *HXT5* was the only hexose transporter expressed and trehalose accumulation continued during this time. Gluconeogenesis with ethanol as precursor has been suggested to be involved in providing glucose for trehalose accumulation after glucose exhaustion in the medium (42). Apparently, no clear relationship exists between *TPS1* expression and a specific hexose transporter. Because *HXT2* was not expressed during trehalose accumulation or *TPS1* expression,

HXT2 is probably not involved in trehalose accumulation. The start of *HXT5* and *HXT7* expression coincided with the initiation of trehalose accumulation and this suggests the involvement of at least one of these gene products in the accumulation of trehalose.

Protein expression of Hxt1p, Hxt3p, Hxt5-HA and Tps1p during batch culture

The protein expression patterns of several hexose transporters were investigated in order to verify the mRNA expression data during batch culture with an initial glucose concentration of 100mM. During the first hours of batch growth, Hxt1p appeared and the protein levels of Hxt1p reached maximum values between 4 and 6 hours after inoculation (figure 2A). The OD600 of the culture increased during that time form 1 at inoculation to 3.1 at t=6h (figure 2B) and the culture grew exponentially at that time. The amount of glucose in the medium decreased during that period from 100mM at t=0h to 77mM at t=6h (figure 2B). After 11 hours of batch growth, Hxt1p was not detected anymore. The extracellular glucose concentration was 1 mM at that time. Hxt3p appeared during the first hour of batch growth and was maximally expressed between 2 and 6 hours after inoculation, which was during the decrease in glucose concentration in the medium from 100mM at t=2h to 77mM at t=6h (figure 2A). Hxt3p levels decreased after six hours of batch growth and the latest time point at which Hxt3p was detected was after 9 hours of batch growth. At this time, the glucose concentration in the medium was 54 mM, but decreased to 2 mM in the next hour. Protein expression of Tps1p was very low in cells grown over night (figure 2A). Tps1p levels increased during the first hours of batch growth. After 5 hours after inoculation, Tps1p levels decreased but the protein remained present. Hxt5p was present during the early hours of batch growth, and this was probably due to the presence of Hxt5p in the preculture. Four hours after inoculation, Hxt5p disappeared and reappeared again after nine hours of batch growth. The amount of Hxt5p increased during later time points, at which extracellular glucose was exhausted.

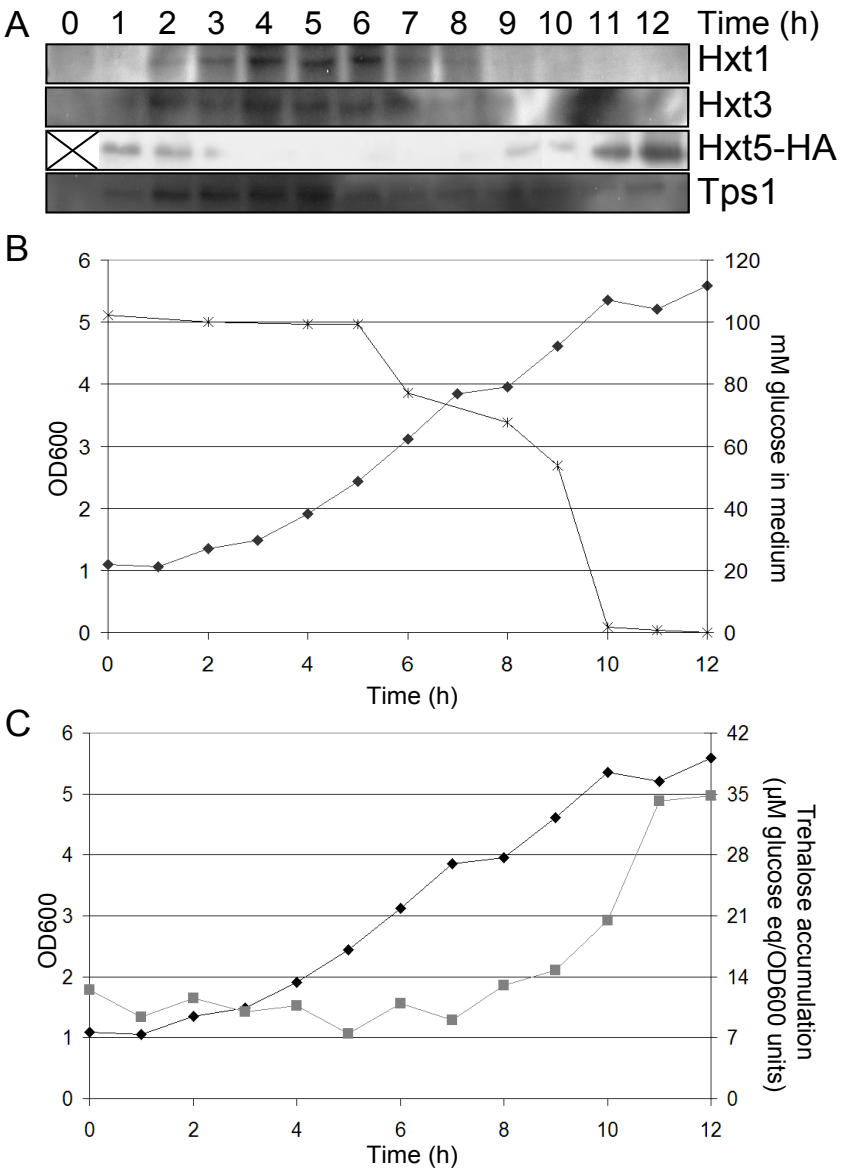

Figure 2. **Trehalose accumulation increased after glucose depletion and correlated with Hxt5p expression.** *HXT5::GFP* expressing cells were inoculated for batch growth in YNB medium containing 2% glucose at t=0h. Cells were collected each hour and protein expression of Hxt1p, Hxt3p, Hxt5p and Tps1p was examined (panel A). The growth of the culture was measured with the use of OD600 determinations (B and C, ♦) and the amount of glucose in the medium (B, x) and the accumulation of trehalose (C, ■) were examined enzymatically as described by Silljé et al., (1).

Trehalose accumulation started later than the presence of the Tps1 protein (figure 2A, C) and this suggest additional regulation of trehalose accumulation besides the presence of Tps1. Over-night grown cells had stored trehalose and this amount of trehalose was decreased by about 25% during the first hour after inoculation. The background level of trehalose storage was maintained as long as the glucose concentration in the medium was present at a concentration above 55 mM (w/v). The glucose concentration decreased rapidly from 54mM at t=9h to 2mM at t=10h, and the amount of glucose stored in trehalose started to increase. The amount of trehalose increased for the next two hours, and remained constant until 18h after inoculation. 24h after inoculation, the amount of trehalose had decreased again to the level of the cells used for inoculation of this batch culture. These data indicate that mRNA levels of *HXT* genes correlated with the protein expression patterns of the corresponding proteins. The protein expression is however shorter than the mRNA expression. This might be caused by the detection limits of the antibodies but also by the absence of the proteins by additional regulation of protein expression. The accumulation of trehalose was only initiated when Hxt5p was present.

Involvement of hexose transporters in trehalose accumulation

To investigate the role of individual hexose transporters on trehalose synthesis, the accumulation of trehalose was determined in strains deleted for one (*HXT1-5*) or two (*HXT6/7)* hexose transporters. The mutant strains and wild type strain were grown in batch culture with an initial glucose concentration of 2% for 24 hours. Figure 3A shows that deletion of the individual hexose transporters did not result in aberrant growth patterns and each mutant strain reached a similar OD_{600} after 24 hours as compared to the wild type strain. Equal amounts of cells were collected to determine the amount of accumulated trehalose (figure 3B). The accumulation of trehalose was affected in all the hexose transporter deletion mutants. Deletion of *HXT1*, *HXT2*, *HXT3*, *HXT4* or *HXT5* resulted in a decrease of trehalose accumulation to 74%, 87%, 80%, 84%, or 82% respectively; deletion of *HXT6*/*HXT7* reduced

the amount of accumulated trehalose to 65% and this deletion gave the most prominent effect. Thus, although deletion of *HXT6/HXT7* decreased the accumulation of trehalose most, all the hexose transporters seemed to be able to provide glucose for of trehalose synthesis.

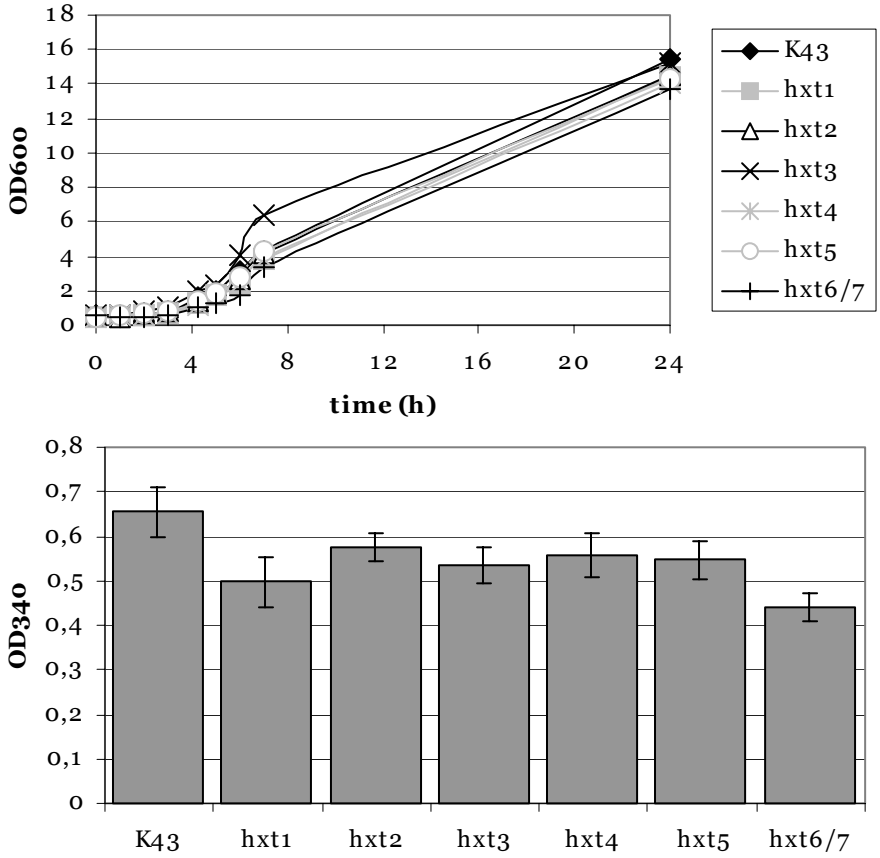

Figure 3. **Influence of individual (*HXT1-5)* or double (*HXT6/7)* deletion of hexose transporters on the accumulation of trehalose after 24 hours of batch culture.** Wild type cells and cells deleted for one or more hexose transporters were grown in batch culture for 24 hours. Growth of the cultures was measured by determination of OD600 (A) and the amount of accumulated trehalose was determined by measurement of OD340 after enzymatic digestion as described by Silljé et al., (1).

Trehalose accumulation correlated with low growth rates in nitrogen-limited continuous cultures on glucose and in galactose-cultured fed batch experiments

Although *TPS1* is required for growth on glucose during all stages of batch growth (43), trehalose was shown in figures 1 and 2 to be only accumulated when the extracellular glucose concentration had decreased below 50mM. The accumulation of trehalose is however also induced during stress conditions when the concentration glucose in the medium is above 50mM. The accumulation of trehalose was shown before to be related to the growth rate of the cells instead of to the extracellular glucose concentration (26). The expression of *HXT5* is also regulated by the growth rate of the cells (14). To investigate the possibility that both trehalose accumulation and *HXT5* expression were induced at the same growth rates, we determined the accumulation of trehalose in nitrogen-limited continuous cultures and took samples at different dilution rates (figure 4A). The extracellular glucose concentration was kept at 200mM and the growth rate of the continuous cultures was regulated by limiting the amount of nitrogen in the medium (39).

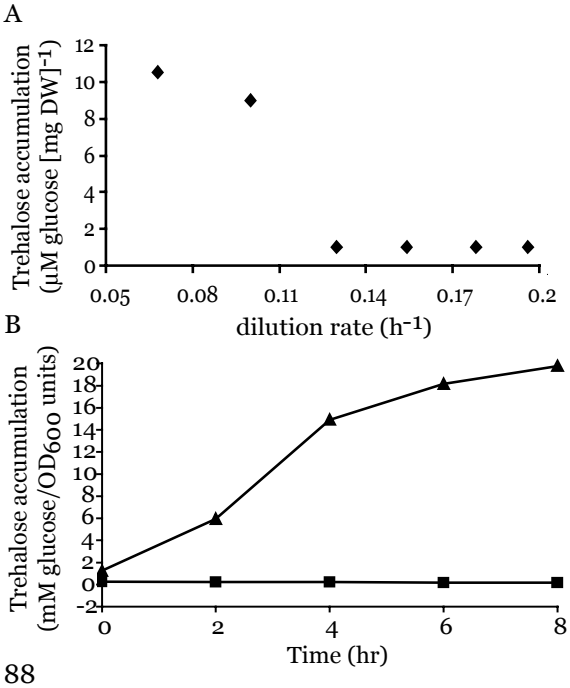

Figure 4. **Trehalose accumulation correlates with the growth rate.** Nitrogen-limited continuous cultures (A) and galactose fed-batch cultures (B) were sampled and trehalose accumulation was determined enzymatically as described by Silljé et al. (1). Fed batch culturing was performed with either 55 fmol galactose cell^{-1} h^{-1} (■) or 10 fmol galactose cell^{-1} h^{-1} (▲).

The major hexose transporters were expressed at similar levels for all the dilution rates tested (data not shown), while *HXT5* was only expressed at the dilution rates 0.075 h^{-1} and 0.1h^{-1} (14). The data presented in figure 4A demonstrate that trehalose was accumulated only at the dilution rates 0.075 h^{-1} and 0.1 h^{-1} and not at the higher growth rates. The expression of the major hexose transporters during the continuous cultures and during the early periods of trehalose accumulation during batch growth made it impossible to exclude a role for the major hexose transporters in trehalose accumulation. However, the dilution rates at which trehalose was accumulated corresponded with the dilution rates at which *HXT5* was expressed. The simultaneous expression of *HXT5* and trehalose synthesis in both batch growth and continuous cultures, suggest a strong correlation between *HXT5* expression and trehalose accumulation.

Induction of a low growth rate can be achieved by fed-batch culturing with low amounts of carbon source in the added medium (1). While *HXT5* is expressed during growth on galactose, the major hexose transporters are not expressed (4) and therefore high and low growth rates on galactose medium were used to investigate whether the correlation between *HXT5* expression and trehalose accumulation could be established. The accumulation of

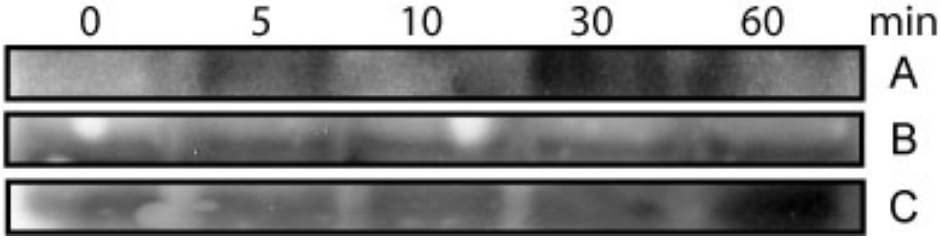

Figure 5. **Hxt5-GFP coimmunoprecipitates with Tps1p after glucose addition to starved cells.** *HXT5::GFP* expressing cells were grown to starvation and glucose was added to a final concentration of 2%. At the indicated time points, samples were collected and Tps1 was immunoprecipitated. Eluates and cell lysates were loaded onto SDS-PAGE gels and after electrophoresis transferred to PVDF membranes. The membrane of the immunoprecipitation (A) was detected with anti-GFP to detect Hxt5-GFP that coimmunoprecipitated with Tps1p. Membranes with lysate samples were detected with anti-Tps1 (B) and anti-GFP (C) to detect respectively Tps1p and Hxt5-GFP in the cell lysates.

trehalose was examined in a fed-batch culture in which the cells were grown at either a high growth rate (achieved by growing cells with a flux of 55 fmol galactose $cell^{-1}$ h^{-1}), or at a low growth rate (achieved by growing cells with a flux of 10 fmol galactose $cell^{-1}$ h^{-1}). Figure 4B shows that during high growth rates trehalose was not accumulated. When cells were grown at a low growth rate, trehalose was rapidly accumulated. We have previously shown that in fed batch cultures with 10 fmol galactose $cell^{-1}$ h^{-1} *HXT5* was expressed but not during growth on 55 fmol galactose $cell^{-1}$ h^{-1} (14). We therefore conclude that the accumulation of trehalose is correlated with expression of *HXT5* in wild-type cells.

Interaction between Hxt5p and Tps1p

Because of the correlation between trehalose accumulation and *HXT5* expression, we investigated a possible physical interaction between Hxt5p and Tps1p. We were not able to coimmunoprecipitate Hxt5p together with Tps1p from stationary phase cells (figure 5, t=0min). Figure 2C already showed that the accumulation of trehalose was restricted to the first 18 hours of batch growth. This might explain the lack of Tps1-Hxt5 interaction in stationary phase cells. Because it was suggested before that trehalose might be transiently accumulated after glucose addition to starved cells (36), we performed the following experiment. Cells expressing *HXT5::GFP* were cultured to stationary phase and subsequently transferred to fresh, 2% glucose containing medium. Samples were collected at intervals and Tps1 was immunoprecipitated. After gelelectrophoresis and Western blotting, the membranes were incubated with anti-GFP to visualize Hxt5-GFP that might have been coimmunoprecipitated with Tps1p. During the stationary phase, Tps1p was present at low levels (figure 2) but rapidly appeared during the first 5 minutes after transfer of the cells to fresh, 2% glucose containing medium. Tps1p remained present in the cells (figure 2). Hxt5p was present in the stationary phase cells and remained present during the first hour (figure 5). An interaction between Hxt5p and Tps1p appeared 5 minutes after transfer of the cells to fresh, 2% glucose containing medium. This interaction was still present at 30 minutes after

transfer of the cells and was lost one hour after transfer of the cells to fresh, 2% glucose containing medium. These data suggest a functional complex of Hxt5-Tps1 after addition of glucose to starved cells.

Hxt5 localized to the endoplasmic reticulum in stationary phase cells

The role of Hxt5p as a hexose transporter requires the presence of this transporter at the plasma membrane. Fluorescence studies of Hxt5-GFP showed a signal that was characterized as plasma membrane labeling after glucose exhaustion in batch growth (11). In order to localize Hxt5p in more detail, we performed electron microscopy on cryosections labeled for Hxt5-HA. Surprisingly, Hxt5-HA showed an intense endoplasmic reticulum (ER) labeling and only a low plasma membrane (PM) labeling (figure 6A, B). The labeled ER was situated just below the plasma membrane, which might explain the supposed plasma membrane localization of Hxt5-GFP (11). Some vacuolar (V) labeling is present as well as labeling of vesicular structures (VS). The labeling of these latter structures might represent the constitutive renewal of Hxt5p at the ER and the plasma membrane (Chapter 6). The mitochondria (M) were not labeled for Hxt5p. The localization of Hxt5-HA at the plasma membrane suggests that Hxt5p can indeed function as a hexose transporter in the stationary phase.

The ER localization of Hxt5-HA suggests however, that Hxt5p has other functions as well during the stationary phase. A possible role for Hxt5p in gluconeogenesis is suggested by analogy with the presence of the glucose transporter GLUT7 in the ER of mammalian cells, which is involved in gluconeogenesis (44).

Discussion

In this study, we investigated the suggested interplay between one or more hexose transporters with the expression of *TPS1* and with the accumulation of trehalose (14, 36). The expression of the hexose transporters and *TPS1* during batch growth revealed no explicit coexpression of one single hexose transporter with *TPS1*. The accumulation of trehalose started when the

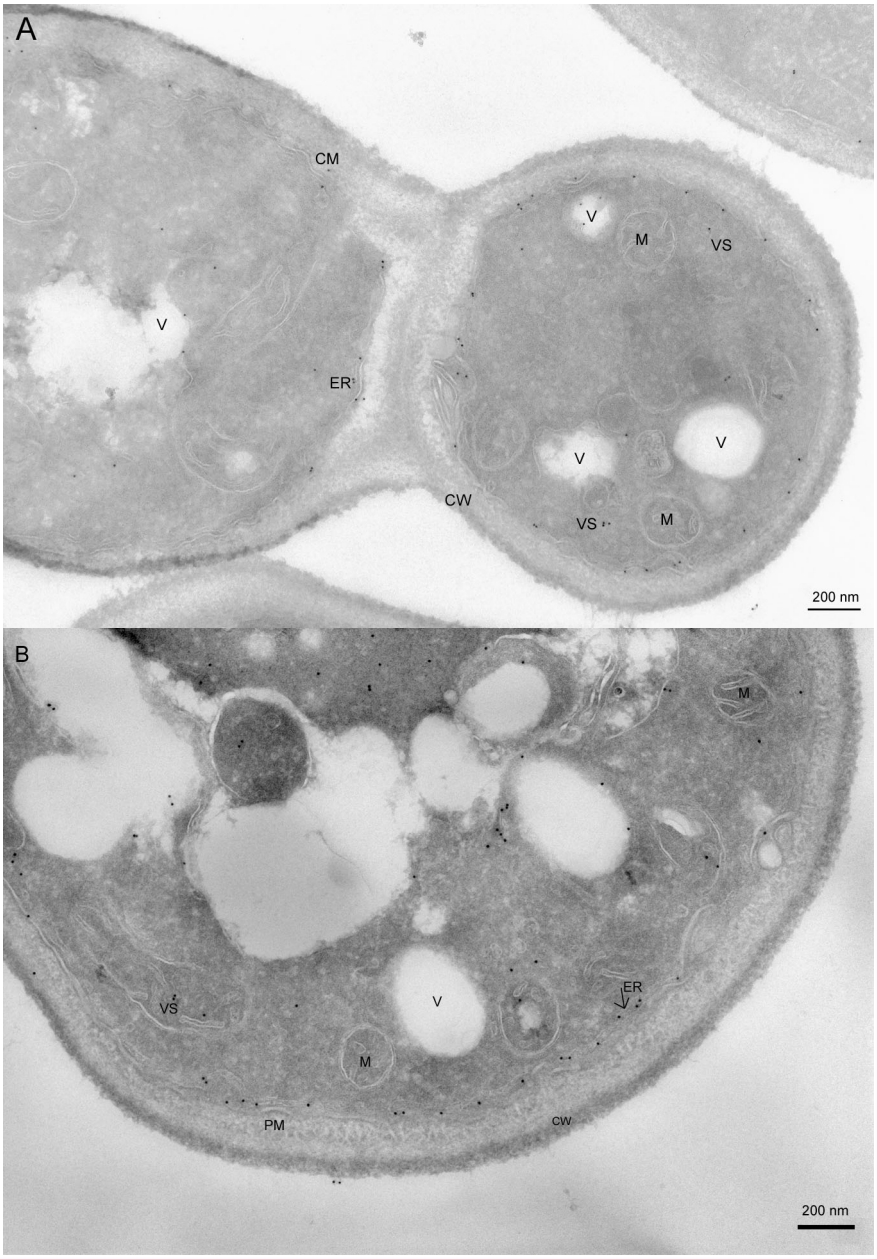

Figure 6. **Electron microscopy of cryosections of starved cell detected for Hxt5-HA.** *HXT5*::*HA* expressing cells were grown to starvation and processed for immunogoldlabeling on cryosections for electron microscopy. Several ultrastructures are indicated in the figure: CM, cell membrane, CW, cell wall, M, mitochondrion; ER, endoplasmic reticulum; V, vacuole; VS, vesicular structure. (A) magnitude 20K, (B) magnitude 25K, scale bars 200 nm.

extracellular glucose concentration was below 50 mM and at this time, *HXT1*, *HXT3*, *HXT4*, *HXT5* and *HXT7* were all expressed, while *HXT2* was not. The expression of the hexose transporters during batch growth on glucose corresponded with the data obtained by Diderich et al (4). The expression of *TPS1* has long been known to be required for exponential growth on glucose (43) and the observed expression of *TPS1* before trehalose accumulation started was thus expected. The continued accumulation of trehalose after glucose depletion together with the expression of *HXT5* as only hexose transporter at that time might suggest a more prominent role for *HXT5* in trehalose accumulation than for the major hexose transporters. Western blot data confirmed the protein expression was parallel to the mRNA expression patterns of *HXT1*, *HXT3, HXT5* and *TPS1*. The accumulation of trehalose started in these experiments several hours after the appearance of Tps1 protein. This suggested an extra regulation in the accumulation of trehalose than only the presence of Tps1p. Trehalose accumulation continued after glucose depletion, when Hxt5p was the only hexose transporter present.

The glucose used to accumulate trehalose during the stationary phase has to be acquired by gluconeogenesis as the extracellular glucose was exhausted (42). Tps1p is required immediately after addition of glucose to starved cells or to cells cultured on other carbon sources than glucose, fructose or mannose. Addition of glucose to *tps1*Δ cells causes a growth arrest by a quick depletion of free phosphate and ATP, together with an increase of early intermediates of the glycolysis (36). The suggested modulation of hexose transport or entry of glucose into glycolysis (29, 33, 36) was strengthened by the interaction between Hxt5p and Tps1p after glucose addition to stationary phase cells. The interaction between Hxt5p and Tps1p suggests in this respect either that trehalose is temporarily accumulated in these cells or that glucose influx rate via Hxt5p is regulated by interaction with Tps1p. In the latter model, a role was suggested for trehalose-6-phosphate in the inhibition of hexokinases. This would lower the rate of glucose phosphorylation and thus the rate of glucose influx into glycolysis. Both options could help cells to cope with the increased concentrations of glucose in the medium by prevention of an overflow of glycolysis. In any case, these models can only function if the

components are in the same complex. We therefore propose that in starved cells, a functional complex consisting of at least Hxt5p and Tps1p, but probably also containing Hxk2p, is required to efficiently respond to glucose addition and to prevent energy exhaustion. The presence of Hxk2p is required to achieve that the early steps of glucose metabolism are carried out as a vectorial process. In addition, deletion of *HXK2* enable a *tps1*Δ strain to grow on glucose (45) and this suggests that Hxk2p is somehow connected to Tps1p function. Because of the suggested role of this complex, we named it the glucose-response complex (GRC). Deletion of either Hxt5p or Hxk2p is not lethal for a cell, because other proteins can take over. For Hxt5p, other hexose transporters might become expressed, and for Hxk2p, Hxk1p or Glk1p might take over. Deletion of *TPS1*, however, causes energy depletion, because no redundant protein is available for activity of the GRC.

The surprising labeling of Hxt5 at the endoplasmic reticulum (ER) is in analogy with the presence of a glucose transporter in the ER in mammalian cells (44). In mammalian cells, the final step of gluconeogenesis, from glucose-6-phosphate to free glucose, is performed in the ER. The resulting glucose is transported out of the ER by GLUT7, which is homologous to glucose transporters in the plasma membrane (44). The labeling of Hxt5p at the ER suggests a role for Hxt5p in gluconeogenesis. Trehalose accumulated after glucose depletion is probably formed from glucose derived by gluconeogenesis. This might explain the simultaneous expression of HXT5 and the accumulation of trehalose. The coimmunoprecipitation of Hxt5p and Tps1p strengthens the suggestion that Hxt5p is directly involved in the accumulation of trehalose. Possibly Hxt5p and Tps1p are also in the same complex under trehalose accumulating conditions during for example batch growth. This might ensure the storage of glucose from gluconeogenesis as trehalose in order to prevent a futile cycle of gluconeogenesis and ethanol production.

The low abundance of Hxt5-HA at the plasma membrane might explain the low concentration of glucose in the medium of ethanol-grown nitrogen-limited continuous cultures (4). This low abundance of Hxt5p at the plasma membrane prevents that cells lose much glucose that was generated via

gluconeogenesis, but still enables cells to quickly respond to glucose addition after depletion.

To summarize, we conclude that Hxt5p is involved in trehalose accumulation and we suggest that the glucose-response complex GRC might prevent an overflow of glucose into the glycolysis by restriction of glucose influx into the cell or into the glycolysis. In this complex at least Hxt5p and Tps1p are present and the GRC is supposed to function after glucose addition to starved cells or to cells grown on other carbon sources than glucose, fructose or mannose. The presence of Hxt5p in the ER during the stationary phase suggests a role for Hxt5p in the gluconeogenesis and Hxt5p might shuttle glucose derived from gluconeogenesis directly to the TPS complex for trehalose accumulation.

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Chapter 5

The role of the Hxt5-Tps1 complex in starved cells

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Abstract

Recently we have demonstrated that Hxt5p interacts with trehalose–6-phosphate synthase (Tps1p) to form the so-called glucose-response complex (GRC). To vectorially shuttle glucose upon influx to either the glycolysis or trehalose synthesis, the hexokinase II (Hxk2p) was also suggested to be included in the GRC. The suggested function of the GRC is to restrict the influx of glucose into glycolysis and thereby to enable cells to express the required genes for growth on glucose, such as the major hexose transporters (*HXT1-4* and *HXT6/7*).Cells that are unable to form this complex, because *TPS1* is deleted, are unable to grow in glucose. These cells rapidly become energy exhausted due to accumulation of early intermediates of glycolysis and depletion of ATP. As Hxt5p is the only hexose transporter expressed in stationary phase cells, we have studied its role in these cells upon stimulation of growth by the addition of glucose to the medium.

The ability to form the GRC did not affect the expression of *HXT5* as this gene was normally expressed on galactose medium in a *tps1*Δ mutant. The GRC is however required for induction of expression of *HXT5* and of *HXT1*, HXT3/4 and *HXT7* during growth on glucose.

Upon addition of glucose to starved cells, the amount of accumulated trehalose was rapidly decreased. We therefore conclude that the GRC restricts the influx of glucose not by transient accumulation of trehalose. Instead, it is suggested that trehalose-6-phosphate inhibits Hxk2p.

Introduction

In *Saccharomyces cerevisiae*, a family of hexose transporter (Hxt) proteins facilitates the transport of glucose into the cell. This family of hexose transporters exists of at least 20 members. Six hexose transporters are considered to be the major hexose transporters, namely *HXT1-4* and *HXT6/7* and their expression is regulated by the extracellular glucose concentration (1).

Expression of *HXT5* was demonstrated to enable growth on glucose in a strain deleted for *HXT1-17* and *GAL2*, although this growth is at a very low rate despite the moderate affinity of Hxt5p for glucose (2). Furthermore, *HXT5*

is only expressed at low growth rates and its expression is independent on the extracellular glucose concentration (3). Hxt5p was suggested to act as a reserve hexose transporter that enables cells to transport glucose when this comes available again after depletion (2).

Cells accumulate the reserve carbohydrates trehalose and glycogen only at low growth rates (4, 5). Both trehalose and glycogen accumulation have a role in survival after nutrient depletion and in stress resistance. Cells deleted for the ability to store either trehalose or glycogen survive comparable periods after nutrient depletion as wild type cells. Cells that are unable to accumulate both trehalose and glycogen lose viability quickly after nutrient depletion (6, 7).

Glucose that is transported into the cell is phosphorylated by hexokinases (encoded by *HXK1*, *HXK2* or *GLK1*) and this yields glucose-6-phosphate (8). Dependent on the conditions of the cells, glucose-6-phosphate enters glycolysis or is used to accumulate the reserve carbohydrates trehalose and glycogen (9-11). Glucose-6-phosphate is converted to glucose-1-phosphate by phosphoglucomutase (encoded by *PGM1* or *PGM2*) (12). Glucose-1-phosphate is then converted into UDP-glucose by UDP-pyrophosphorylase (encoded by *UGP1*) (13). UDP-glucose can be used to form trehalose by action of the trehalose synthase complex. This multimer complex consists of Tps1p, Tps2p, Tps3p and Tsl1p. Trehalose-6-phosphate synthase (encoded by *TPS1*) converts UDP-glucose together with glucose-6-phosphate into trehalose-6-phosphate (14, 15). Trehalose-6-phosphase phosphatase (encoded by *TPS2*) dephosphorylates trehalose-6-phosphate, which yields trehalose and free phosphate (16, 17). *TPS3* and *TSL1* probable encode regulatory proteins that stabilize the complex and regulate its activity (14, 18, 19).

Deficiency in trehalose accumulation makes it impossible for cells to grow on glucose, fructose or mannose, which are all transported by Hxt proteins. *tps1*Δ cells grown on other carbon sources than glucose, fructose or mannose are viable but are sensitive for glucose (20, 21). Addition of glucose to *tps1*Δ cells results in a rapid depletion of ATP and a hyperaccumulation of early intermediates of the glycolysis, especially fructose1,6-bisphosphate (20-24).

This rapid overflow of the glycolysis is not caused by lack of capacity of the glycolysis, as cells growing in the exponential phase on galactose medium have sufficient glycolysis capacity, but by a lack of regulation of glucose influx into glycolysis (25). It has been suggested that under normal conditions, Tps1p or its product, trehalose-6-phosphate, restricts the influx of glucose into the cell by interaction with hexose transporters or into the glycolysis by modulation of the hexokinases (14, 19, 22). The growth defect of *tps1*Δ strains is relieved by additional deletion of *HXK2* (26), which reduces the hexokinase activity of the cell. We have shown in the previous chapter that Hxt5p and Tps1p coimmunoprecipitate after glucose addition to starved cells. We proposed the presence of the glucose-response complex (GRC), which is a functional complex consisting of at least Hxt5p and Tps1p, possibly together with Hxk2p. The GRC is thought to be required to efficiently respond to glucose addition and to prevent energy exhaustion after glucose addition.

In this report, we provide evidence that Hxt5-Tps1 indeed functions in a complex to enable cells to cope with fresh glucose addition after glucose depletion. *tps1*Δ cells demonstrated normal *HXT5* expression during growth on galactose, showing that the expression of *HXT5* was not dependent on *TPS1* or the GRC. Expression of *HXT5* was continued after glucose addition when the cells were first grown to starvation on galactose. When *tps1*Δ cells were grown to the exponential phase on galactose, *HXT5* and *HXT1-4* plus *HXT7* were not expressed. This indicates that the proposed complex is not functional when *TPS1* is deleted. The GRC functions in wild type cells probably by inhibition of Hxk2p by trehalose-6-phosphate, as transient trehalose accumulation after glucose addition was not observed.

Materials and Methods

Strains, media and growth conditions

The *Saccharomyces cerevisiae* strains used in this study are indicated in Table 1. Cells were grown at 30°C at 180 rpm in a shaking incubator (New Brunswick Scientific, Edison, NJ, USA) in 0.67% (w/v) yeast nitrogen base without amino acids (Difco, Sparks, MD, USA) and 2% (w/v) of the carbon

source as indicated in the text. For the JBY20 strain, uracil was added to a final concentration of 2 μg/ml.

Table 1: List of strains used:

Yeast strain	Genotype	Source
CEN.PK 113-7D (wild type)	MATa SUC2 MAL2-8^c	P. Kötter (Frankfurt, Germany)
CEN.PK 113-3C Δtps1	MATa SUC2 MAL2-8^c tps1::TRP1	(6)
JBY20 (Hxt5-HA)	MATa, SUC2, MAL2-8^c, *ura3*, HXT5::HA	J. Becker (Düsseldorf, Germany)

Northern blot analysis

Total RNA was isolated, separated on agarose gels and transferred to Hybond-N membrane (Amersham Pharmacia Biotech, Buckinghamshire, United Kingdom) as described earlier (3). Oligonucleotides directed against *HXT1-7* (27) were labelled as described (3). A probe against *U2* was obtained by PCR, using plasmid pRS313U2 as template and primers 5'-GTTTTGCAAGGAAAGG-3' and 5'-CGAGAGAAACATCAAGCG-3'. The probe was labelled with [α-^{32}P]dCTP using prime-a-gene labelling system according to manufacturer's protocol (Promega, Madison, Wisconsin, USA). Hybridization and washing was performed as described earlier (3).

Real-time quantitative PCR

Total RNA was isolated as described by Verwaal et al. (3). To ensure similar amounts of starting materials, total RNA concentrations were carefully measured with a Nanodrop ND-1000 Spectrophotometer (Nanodrop Technologies Inc., Wilmington, DE, USA). To 1 mg of total RNA, 1 ng 1.2kb kanamycin positive control RNA (Promega, Wisconsin, USA) was added. Next, chromosomal DNA was degraded by treatment with DNaseI (amplification grade, Invitrogen, Carlsbad, CA, USA) according to manufacturer's

instructions. Subsequently, cDNA was amplified using Omniscript RT kit (Qiagen, Hilden, Germany) with the following modifications: a 10mM dNTP stock (Promega) and an Oligo-dT18 primer (Isogen Life Science, IJsselstein, The Netherlands) were used in the reactions. Pipetting was performed by a pipetting robot (Corbett Robotics, Mortlake, NSW, USA), using the amplified cDNA, qPCR primers as indicated in Table 2 and ABsolute QPCR SYBR Green Mix (Westburg, Leusden, The Netherlands). These qPCR primers were designed using Primer3 (28). The qPCR reactions were performed in a Rotor Gene RG-3000 real-time PCR machine (Corbett Research, Mortlake, NSW, USA). To measure the relative changes in gene expression of HXT5, expression of the housekeeping gene TDH3 was used as reference. The results are presented as ratios of gene expression between the target gene (gene of interest) and the reference gene (TDH3) (29).

Table 2: qPCR primers:

Gene	Forward primer	Reverse primer
TDH3	CGGTATGGCTTTCAGAGTCC	CAACAGCGTCTTCGGTGTAA
HXT5	GGGCGTTGGTGGTATTACAG	ATGGTACCCTCCATTGGACA
TPS1	TGAGCGATGTTTGTTTGGTC	CATCGGTGTTCCAAGGATTT

Results

Effect of TPS1 deletion on the expression of HXT5 during culturing on galactose

In the previous chapter, we demonstrated the presence of Hxt5p and Tps1p in the same complex after glucose addition to starved cells and named this complex GRC for glucose-response complex. To further elucidate the role of the GRC, we investigated whether the ability to form this complex affected the cell's capacity to express *HXT5*. We examined the expression of *HXT5* in a wild type and a *tps1*Δ strain, which is unable to accumulate trehalose. Wild type cells and *tps1*Δ cells demonstrated the same *HXT5* expression pattern when the strains were cultured on galactose to the exponential phase and

transferred to fresh galactose medium (figure 1A). 6 hours after the cells were transferred to fresh galactose medium, the cultures grew exponentially and both strains showed no *HXT5* expression: after 12 hours, both strains exhibited *HXT5* expression and this expression was further increased 24 hours after the cells were transferred to fresh galactose medium. At the latest time point, the cells had reached the stationary phase. These data indicate that the expression of *HXT5* is not dependent on the presence of the *TPS1* gene and therefore not on the presence of the GRC.

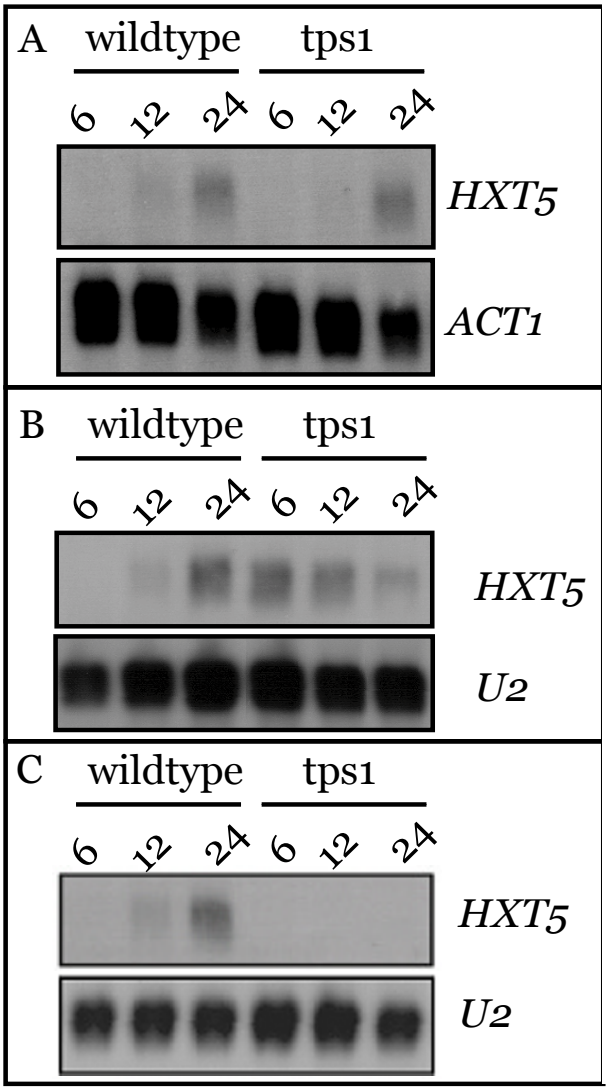

Figure 1. ***HXT5* expression in wild type and *tps1*Δ cells during growth on galactose, and after transfer from galactose to glucose medium.** Wild type cells and *tps1*Δ cells were grown to the exponential phase on galactose medium and transferred to fresh, 2% galactose containing YNB medium (A). Wild type cells and *tps1*Δ cells were grown on galactose to either the stationary phase (B) or exponential phase (C) and transferred to 2% glucose containing YNB medium. At the indicated time points, cells were collected and mRNA was isolated for Northern blot detection.

Effect of TPS1 deletion on the expression of HXT5 during culturing on glucose

The *tps1*Δ strain fails to grow on glucose, fructose and mannose, and addition of one of these carbon sources to *tps1*Δ cells grown on galactose, causes a growth arrest (20-24). Because addition of glucose to *tps1*Δ cells caused immediate growth arrest and a low growth rate is an inducer of *HXT5* expression, we investigated whether the expression of *HXT5* was induced in the *tps1*Δ mutant upon glucose addition. Wild type and the *tps1*Δ strain were cultured on galactose to the stationary phase, and then transferred to fresh, 2% glucose containing medium. The wild type strain exhibited the same expression pattern as during continued growth on galactose, *i.e.* no *HXT5* expression 6 hours after transfer to fresh glucose medium, low expression of *HXT5* 12 hours after transfer and high *HXT5* expression in the stationary phase (figure 1B). The *tps1*Δ strain however exhibited *HXT5* expression on 6, 12 and 24 hours after transfer to fresh glucose medium (figure 1B) while there was no increase in OD_{600} (data not shown). The expression of *HXT5* in the *tps1*Δ mutant was at a maximum at 6 hours after transfer of the culture to fresh glucose medium and decreases at 12 and 24 hours after transfer. These data suggest that the lack of growth indeed induced *HXT5* expression in the *tps1*Δ strain. On the other hand, the decrease in *HXT5* expression in time in the *tps1*Δ strain indicated that the observed expression might be a remnant of the *HXT5* expression in the preculture.

To investigate whether the observed *HXT5* expression in *tps1Δ* cells after transfer to glucose medium as shown in figure 1B was a remnant of *HXT5* expression in the preculture or *de novo* expression, the following experiment was performed. Wild type cells and *tps1*Δ cells were grown to the exponential phase on galactose medium and subsequently transferred to glucose medium. The wild type strain demonstrated the same *HXT5* expression pattern as described for figure 1A and 1B (figure 1C). The *tps1*Δ strain on the contrary totally lacked *HXT5* expression on 6, 12 and 24 hours after transfer (figure 1C) while the OD_{600} did not increase. These data indicate that the *tps1*Δ strain was

not able to express *HXT5* on glucose in spite of the absence of growth and that the expression of *HXT5* demonstrated after preculture on galactose to the stationary phase was a remnant of the *HXT5* mRNA present during the stationary phase and did not represent newly synthesized mRNA molecules.

Quantitative measurements of HXT5 expression

To further investigate the suggestion that *HXT5* expression in *tps1*Δ cells is not possible during growth on glucose, we performed real-time quantitative PCR to detect *HXT5* mRNA from samples cultured as described above (figure 2). *HXT5* expression was determined in wild type and *tps1*Δ strains after preculture on galactose to the exponential or stationary phase, and after 6 and 24 hours after transfer of the cells to fresh, glucose containing medium (figure 2). As expected, the wild type strain demonstrated very low *HXT5* expression during the exponential phase during preculture on galactose and elevated *HXT5* expression in the stationary phase after preculture on galactose. During the exponential phase on glucose (6 hours after transfer) wild type cells from both preculture (exponential or stationary phase) conditions showed no *HXT5* expression. 24h after transfer to fresh, glucose medium, wild type cells exhibited induced *HXT5* expression independent of their preculture conditions. The *HXT5* expression in the stationary phase after transfer to glucose medium was however higher when cells were cultured to the stationary phase than when they were cultured to the exponential phase on galactose. Thus, while the conditions at 24 hours after transfer to fresh medium were comparable, the preculture conditions determined the quantitative amount of *HXT5* expression.

The *tps1*Δ mutant showed no *HXT5* expression during the exponential phase on galactose, which was comparable with the wild type cells. When the *tps1*Δ cells, precultured on galactose to the exponential phase, were transferred to glucose, *HXT5* expression was not induced at 6h or at 24h after transfer to glucose medium. The *tps1*Δ cells did not grow at all under these conditions and therefore the absence of *HXT5* expression was not a result from high growth rates. When the *tps1*Δ mutant was grown to the stationary phase on galactose,

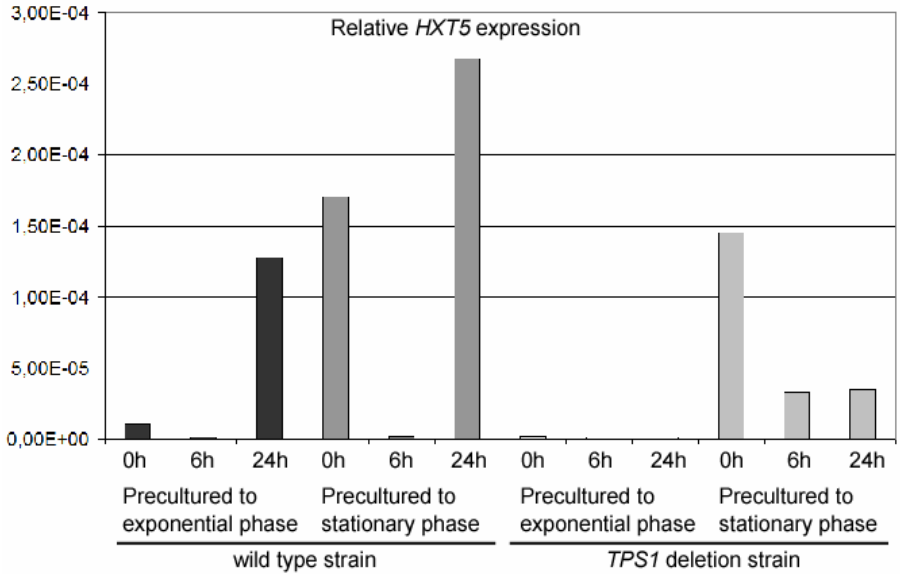

Figure 2. **Relative expression of *HXT5*.** Wild type and *tps1*Δ cells were grown to the stationary or exponential phase on galactose medium and subsequently transferred to 2% glucose medium. At the indicated time points after transfer, aliquots of cells were collected and mRNA was isolated. After cDNA synthesis, qPRC was performed.

these cells exhibited enhanced *HXT5* expression. The *tps1*Δ cells expressed comparable *HXT5* levels as the wild type cells during the stationary phase on galactose. When the galactose grown stationary phase *tps1*Δ *cells* were transferred to fresh, glucose containing medium, they exhibited *HXT5* expression at 6 hours after the transfer to glucose medium in contrast to the wild type cells. 24 hours after transfer to fresh glucose medium, the *tps1*Δ cells exhibited *HXT5* expression, but only at the level found at 6 hours. The level of *HXT5* expression in the wild type strain was approximately 50% higher at the stationary phase after glucose preculture than in the initial stationary phase of the preculture on galactose; in the *tps1*Δ deletion strain the *HXT5* expression was decreased to approximately 25% 24h after transfer to glucose medium as compared to the level of *HXT5* at the stationary phase after galactose growth. Because also the housekeeping genes that were used as references in these experiments decline severely in the *tps1*Δ strain after transfer to glucose

medium, we suggest that the observed *HXT5* signal in *tps1*Δ cells after transfer to glucose medium is indeed a remainder of the signal in the stationary phase culture. Taken together, these data suggest that the *tps1*Δ strain is normally able to express *HXT5* during growth on galactose but is unable to do this during growth on glucose.

Effect of TPS1 deletion on expression of HXT1, HXT3/4 and HXT6/7

Efficient response to glucose by the GRC includes induction of expression of major hexose transporters. This induction of expression might be an indirect effect, as prevention of energy depletion might enable other regulatory systems to induce the expression of the hexose transporters. Because the *tps1*Δ strain is unable to grow on glucose and the regulation of *HXT5* was absent on glucose containing medium, we wondered whether the expression of the major hexose transporters was also disturbed. Therefore, we

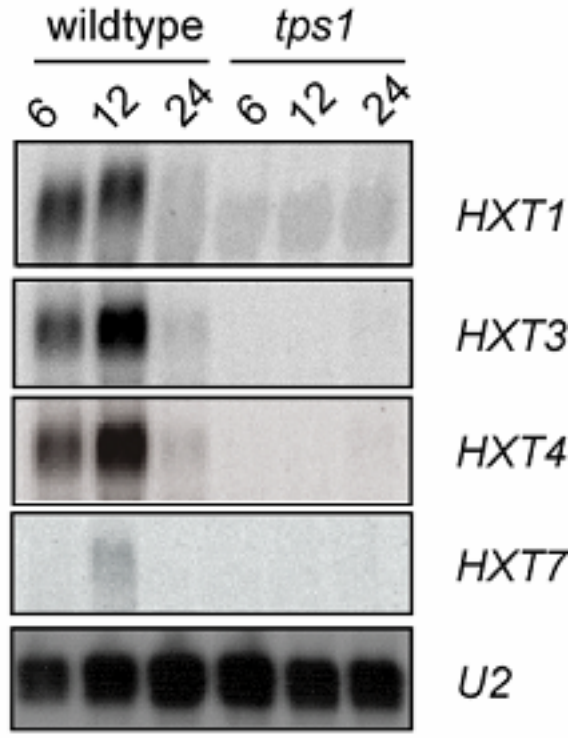

Figure 3. ***HXT1-4* and *HXT7* expression in wild type and *tps1*Δ cells after transfer from galactose to glucose medium.** Wild type cells and *tps1*Δ cells were grown on galactose medium to the exponential phase and transferred to 2% glucose containing YNB medium. At the indicated time points, cells were collected and mRNA was isolated for Northern blot detection.

investigated the expression of *HXT1*, *HXT3*, *HXT4* and *HXT6/7* in both wild type and *tps1*Δ strains, which were precultured to the exponential phase on galactose and subsequently transferred to fresh, glucose containing medium (figure 3). None of the major hexose transporters was expressed in the exponential phase on galactose medium (data not shown). In wild type cells, *HXT1*, *HXT3* and *HXT4* were expressed at 6 hours after transfer to glucose medium. Their expression was increased at 12 hours after supply of the new medium and no expression was present anymore at 24 hours. *HXT7* was not expressed at 6 hours after transfer of the wild type cells to fresh, glucose containing medium. 12 hours after the transfer *HXT7* mRNA was present and this expression was decreased at 24 hours after the addition of fresh, glucose containing medium. The expression data of the major hexose transporters are consistent with the data presented in figure 1 of the previous chapter. In the *tps1*Δ strain however, none of the hexose transporters examined was expressed. At 6, 12 and 24 hours after transfer of the cells to fresh, glucose containing medium, *HXT1*, *HXT3*, *HXT4* and *HXT7* were not expressed. Therefore, the expression of *HXT1*, *HXT3*, *HXT4*, *HXT5* and *HXT7* is dependent on the presence of *TPS1* during growth on glucose. Thus, disruption of the GRC by deletion of *TPS1* makes it impossible for cells to cope with glucose.

Trehalose is rapidly degraded after glucose addition to starved cells

The way of GRC function was next investigated. It was proposed before that such a complex would either use trehalose-6-phosphate to inhibit hexokinases or trehalose accumulation to restrict the influx of glucose into glycolysis (22). To investigate the way of GRC function to prevent energy depletion, the amount of trehalose was investigated after glucose addition to starved cells. In figure 4, the levels of trehalose are shown during a short time after glucose addition. The amount of glucose that was stored as trehalose was lowered already after two minutes after glucose addition to starved cells and continued for five minutes. This level was kept constant at ten and fifteen

minutes after addition of glucose to starved cells. The influx of glucose into glycolysis is thus not regulated by synthesis of trehalose.

Taken together, these data presented in this study indicate that the GRC functions by inhibition of hexokinases by trehalose-6-phosphate. The GRC is probably composed of Hxt5p, Tps1p and a hexokinase. Disruption of the GRC by deletion of *TPS1* leaves the complex functionally inactive and cells are then not able to respond to glucose.

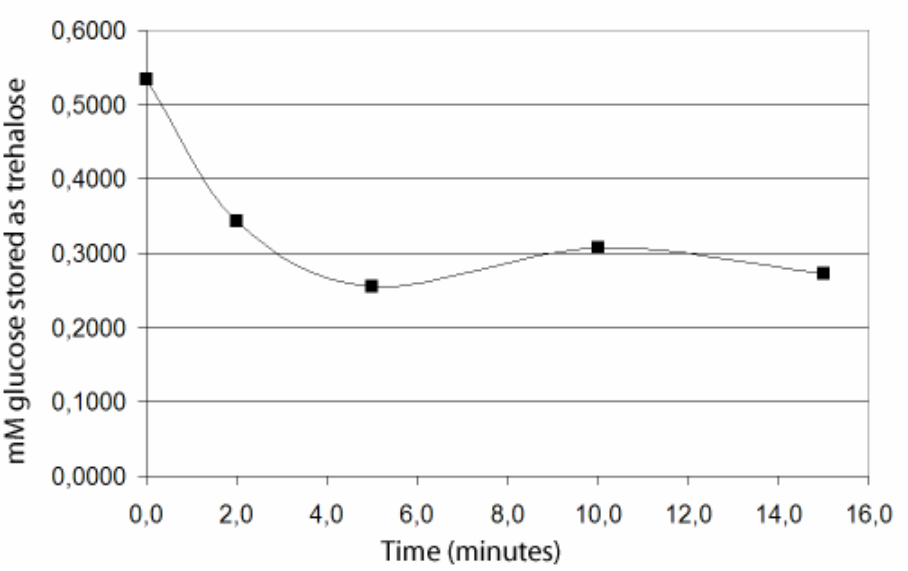

Figure 4. **Trehalose is rapidly degraded after glucose addition to starved cells.** Cells were inoculated in batch culture and cultured for 48 hours. Glucose was added to a final concentration of 2% and at the indicated time points, samples were collected. The amount of glucose that was stored as trehalose is shown.

Discussion

The presence of Hxt5p and Tps1p in the same complex after glucose addition to starved cells led to the model of the glucose-response complex (GRC) described in Chapter 4. Besides Hxt5p and Tps1p, we proposed that also Hxk2p is involved in the GRC in order to acquire a vectorial process of the early steps of glucose metabolism (8, 12, 13). This complex ensures an efficient

response to glucose addition by prevention of an overflow of glycolysis and thus by prevention of energy exhaustion. In order to explore the action of the GRC in more detail, the effect of disruption of the GRC was studied. The expression of *HXT5* in the *tps1*Δ mutant was similar to the expression in the wild type strain during growth on galactose an the expression of *HXT5* was thus not dependent on the GRC. However, the expression of *HXT5* differed between the wild type and the *tps1*Δ strains when they were cultured on glucose medium. The expression of *HXT5* in the wild type strain on glucose medium was similar to that on galactose medium. *tps1*Δ cells transferred to glucose medium after being grown to the stationary phase on galactose showed *HXT5* mRNA, while no *HXT5* was induced when the *tps1*Δ cells were precultured to the exponential phase on galactose before transfer to glucose containing medium. This indicated that *tps1*Δ cells on glucose were not capable of inducing *HXT5* expression in spite of the absence of growth, and that the *HXT5* expression in the *tps1*Δ cells from the stationary phase on galactose medium was a remnant of the *HXT5* expression in the preculture. This assumption was further strengthened by the real time quantitative PCR data, which showed a decrease in *HXT5* expression levels when *tps1*Δ cells precultured to the stationary phase on galactose were transferred to glucose medium. The expression of *HXT5* was therefore not dependent on the presence of the *TPS1* gene during growth on galactose, but it was during growth on glucose.

The inability of the *tps1*Δ strain to grow on glucose seems to be related to the inability to express any hexose transporter as also the major hexose transporters were not expressed in the *tps1*Δ cells. In a broader perspective, the inability of the *tps1*Δ strain to express any of the hexose transporters examined might be caused by the energy depletion that is produced by the addition of glucose to these cells (20-24), which renders it impossible for the *tps1*Δ cells to do anything at all after energy depletion is reached. Additional deletion of the hexose kinase II gene (*HXK2*) enable growth on glucose of a *tps1*Δ strain (26). This indicated that glucose addition itself is not toxic to cells, but the lack of regulation caused the energy depletion. The disruption of the

GRC leads thus to energy depletion because of the lack of regulation of influx of glucose into glycolysis.

Both trehalose accumulation and inhibition of Hxk2p by trehalose-6-phosphate would decrease the influx of glucose into glycolysis (14, 19, 22). Because the levels of accumulated trehalose dropped within minutes after glucose addition to starved cells, we conclude that the GRC regulates the influx of glucose into glycolysis by inhibition of Hxk2p via trehalose-6-phosphate. A schematic model of the different components of the GRC and metabolic intermediates is presented in figure 5.

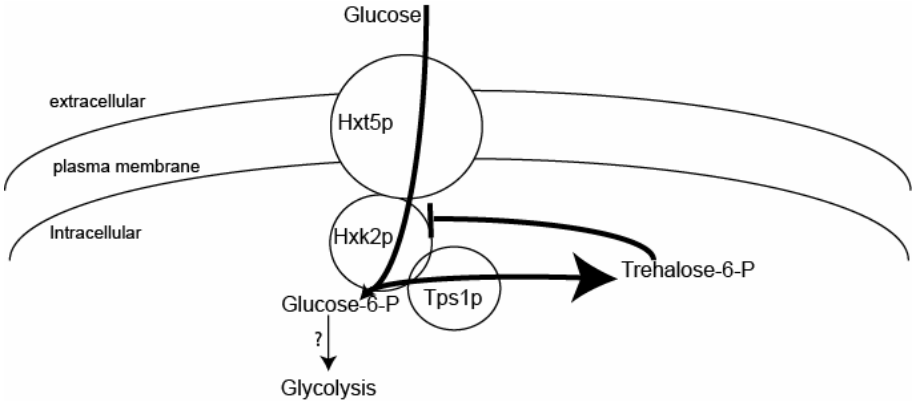

Figure 5. **Model of GRC.** Glucose is transported into the cell via Hxt5p. Glucose is directly phosphorylated by Hxk2 to glucose-6-phosphate. Glucose-6-phosphate can be shuttled to either the glycolysis or via different intermediates transformed into trehalose-6-phosphate. Trehalose-6-phosphate can inhibit Hxk2 to phosphorylate glucose. The suggested vectorial process is then stopped and no extra glucose can be transported in to the cell.

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Chapter 6

Degradation of the hexose transporter Hxt5p in *Saccharomyces cerevisiae*

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Abstract

Hxt5p is a member of a multigene family of hexose transporter (Hxt) proteins which translocate glucose across the plasma membrane of the yeast *Saccharomyces cerevisiae*. In contrast to other major hexose transporters of this family, Hxt5p expression has been demonstrated to be regulated by the growth rate of the cells and not by the external glucose concentration Further, Hxt5p is the only glucose transporter expressed during stationary phase. These observations suggest a different role for Hxt5p in *S. cerevisiae*. Therefore, we have studied the metabolism and localization of Hxt5p in more detail. Inhibition of *HXT5* expression in stationary phase cells by addition of glucose, which decreases the growth rate, leads to a decrease in the amount of Hxt5 protein within a few hours. Addition of glucose to stationary phase cells resulted in a transient phosphorylation of Hxt5p on serine residues, but no ubiquitination could be measured. The decrease in Hxt5p levels is caused by internalization of the protein as observed by immunofluorescence microscopy. In stationary phase cells, Hxt5p is localized predominantly at the cell periphery, and upon addition of glucose to the cells the protein translocates to the cell interior. The internalized Hxt5-HA protein is most probably localized to endocytic structures and the vacuole, as the constitutive Hxt5-HA renewal in stationary phase cells was demonstrated with electron microscopy to occur via this pathway. These results suggest that internalization and degradation of Hxt5p in the vacuole occur in an ubiquitination independent pathway.

Introduction

In *Saccharomyces cerevisiae*, glucose is transported into the cell by hexose transporters, which are localized in the plasma membrane (1-5). The family of hexose transporters consists of 20 members of which *HXT1-4* and *HXT6-7* are the major hexose transporters. Expression of one of these transporters allows growth on glucose in the MC996 background in which *HXT1-7* are deleted (6). The expression of the major hexose transporters is mainly regulated by the extracellular glucose concentration (5). In addition, overexpression of each of the genes *HXT5, HXT8-11* or *HXT13-17* in an *hxt1-17*

gal2 deletion strain in the CEN.PK background allows growth on glucose as well (7), indicating that these Hxt proteins are also capable of transporting glucose. Under normal conditions however, the expression of these genes is low except for *HXT5, HXT8* and *HXT13* (5).

The Hxt proteins are highly homologous with 12 transmembrane domains with both the amino- and carboxyterminal tails localized in the cytoplasm (3). The lowest homology is found in the aminoterminal intracellular parts. The largest protein is Hxt5p, which has 592 amino acids, and this is approximately 20 residues longer than the major hexose transporters (8). This difference is located mainly in the aminoterminal intracellular part of the protein, which is 82 amino acids long while that of the major hexose transporters contains between 50 and 65 amino acids.

Due to the differences in protein structure, it is tempting to suggest that Hxt5p has a different function in the cells as compared to the major hexose transporters. The *HXT5* (YHR096c) gene is localized to chromosome III, as are *HXT1* and *HXT4*. As for the other *HXT* genes, deletion of *HXT5* is not lethal to the cells and no clear phenotype results from it (6, 9). *HXT5* expression has been demonstrated to be regulated by the growth rate and its expression is controlled by STRE and HAP elements in the *HXT5* promoter (10). Because of the involvement of the STRE elements under stress conditions, and of HAP elements under glucose limitation and exhaustion, the phenotype of growth-rate regulated *HXT5* expression could therefore result from the action of both STRE and HAP elements depending on the environmental conditions. *HXT5* expression is not strictly controlled by glucose repression as in nitrogen-limited continuous cultures, the expression of *HXT5* is dependent on the growth rate while the glucose concentration in the medium was constant (8). Besides, it is published that the deletion of hexokinase II (*HXK2*) did not derepress *HXT5* expression under high glucose condition while it does derepress the high-affinity glucose transporters *HXT2* and *HXT7* (11). We also suggested a role for Hxt5p in trehalose accumulation next to its function as a hexose transporter (8). As compared to wild-type cells, an *hxt5* mutant strain exhibits a relatively long lag phase before starting exponential growth when glucose is re-administered after glucose depletion as

(9), suggesting that Hxt5p plays a role in adaptation to growth conditions with glucose as carbon and energy source. Under inducing conditions, Hxt5-GFP demonstrated a plasma membrane localization while some label was present in the cytoplasm (9).

We show here that in stationary phase cells, Hxt5-HA is present in both the mother and the developing daughter cell. After increasing the growth rate, the new buds were not labeled anymore and with ongoing time fewer cells were labeled. The label present in stationary phase cells was located at the cell periphery near the plasma membrane. After increase of the growth rate, immunofluorescence demonstrated that Hxt5-HA became localized inside the cells, probably at endocytic vesicles and in the vacuole. Furthermore, Hxt5p was transiently phosphorylated on serine residues after addition of glucose to the stationary phase cells, with a maximum after 30 minutes. No ubiquitination of Hxt5p was however detected. We therefore suggest that the phosphorylation of Hxt5p serves a regulatory role instead of an internalization signal. It is concluded that while the expression of *HXT5* is different from that of the major hexose transporters, the degradation occurs via similar pathways but is regulated in a different way.

Materials and Methods

Strains, media and growth conditions

The yeast strains used in this study were wild-type CEN.PK 113-7D (MAT**a**, *SUC2*, *MAL2-8*^{c}), kindly provided by P. Kötter (Institut für Mikrobiologie, Johann Wolfgang Goethe-Universität, Frankfurt am Main, Germany) and JBY20 (Mat**a**, *SUC2*, *MAL2-8*^{c}, *ura3*, HXT5::HA) by J. Becker (Institut für Mikrobiologie, Heinrich-Heine-Universität, Düsseldorf, Germany). Yeast cells were grown in batch culture on 0.67% (w/v) yeast nitrogen base without amino acids (Difco, Sparks, MD, USA) and 2% glucose, supplemented with 2 μg/ml uracil for the JBY20 strain. Cells were grown at 30°C in a shaking incubator at 180 rpm (New Brunswick Scientific, Edison, USA). Cells were grown for 48h to stationary phase for growth experiments, a

portion of the cells was transferred to fresh, 2% (w/v) glucose containing YNB medium at an OD_{600} of 0.4 (±0.05).

Western Blot analysis

Analysis of proteins with Western blot was performed as described by Verwaal et al (2002), with the modification that samples were centrifuged after collection, washed with ice-cold water and stored at -20°C until use. Furthermore, RIPA buffer (150 mM NaCl, 20 mM Tris-HCl pH 7.5, 1 mM EDTA, 1%SDS and 0.1% Triton X-100) was used as lysis buffer. For detection, PVDF membranes (Roche Diagnostics, Basel, Switzerland) were incubated with anti-HA antibody 12CA5 (Roche) and peroxidase-conjugated rabbit-anti-mouse (Jackson Immunoresearch, Cambridgeshire, United Kingdom). Proteins were visualized with Enhanced Chemiluminescence (Renaissance, NEN Life Science Products, Inc., Boston, MA, USA). The degradation of Hxt5-HA was quantified by dividing the optical density of the signal for Hxt5-HA by the signal for actin on the same PVDF membrane, and the standard errors of the mean (n=5) were calculated and presented in the figure. Measurements were performed on a Fluor-S MultiImager (BioRad, Hercules, CA, USA) and calculations with Microsoft Office Excel.

Immunofluorescence microscopy on whole cells

2.5 * 10^6 yeast cells were fixed by adding an equal volume of 8% formaldehyde and incubated for one hour. Cells were collected by centrifugation at 1500 rpm for 5 minutes. Cells were washed once with 1 ml fresh medium and incubated for 5 minutes with DET (0.1 M dithiothreitol, 0.02 M EDTA and 0.02 M Tris-HCl, pH 8.1). After collecting the cells by centrifugation, the cell pellet was suspended in 0.9 M sorbitol /PBS pH 7.4 (137 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4). Cell walls were digested by adding 10 µl zymolyase (5 mg/ml, Seikagaku Corp., Tokyo, Japan) and incubated for 30 min at 30°C. Spheroplasts were washed with 0.9 M sorbitol/PBS and permeabilized with 0.9 M sorbitol/PBS/1% Triton X-100. After two washes with 0.9 M sorbitol/PBS, cell suspensions were divided over

poly-L-lysine (Sigma Chemical Company, St.Louis, MO, USA) coated cover slips and allowed to settle for 30 min at room temperature. The excess fluid was aspirated off and the coverslips were air-dried for 5 minutes. The coverslips were blocked with PEM (0.1 M Pipes, pH 6.9, 1 mM EGTA, 0.1 mM $MgSO_4$)/1% BSA/0.1% NaN_3 and subsequently labeled with anti-HA 12CA5 (Roche). Cells were washed with PEM/1% BSA/0.1% NaN_3 and incubated in the dark with goat-anti-mouse Cy3 (Jackson Immunoresearch, Cambridgeshire, United Kingdom) in PEM/1% BSA/0.1% NaN_3. Finally, the cells were washed three times and embedded in moviol/DAPI (12). Images were collected using a Nikon digital camera DXM 1200 with a Zeiss Plan neofluar 40x objective (Zeiss, Oberkochen, Germany) and processed using Photoshop 5.5.

Electron microscopy on cryosections

Cells were fixed for 15 min at 30°C and 180 rpm by adding an equal volume of two times concentrated fixative (4% formaldehyde, 0.4% glutaraldehyde in 0.1 M PHEM buffer (60 mM Pipes, 25 mM Hepes, 10 mM EGTA and 2 mM $MgCl_2$), pH 6.9). Cells were collected by centrifugation for 5 minutes at 4000 rpm and fixation was continued for 45 min at room temperature with fresh fixative (2% formaldehyde, 0.2% glutaraldehyde in 0.1 M PHEM buffer). Then the cells were washed with 0.1 M PHEM buffer, and resuspended in 1% formaldehyde in 0.1 M PHEM buffer and stored at 4°C until further use. The cells were washed three times with 0.1 M PHEM buffer and incubated for one hour at room temperature in 1% periodic acid in 0.1 M PHEM buffer. Subsequently, the cells were washed three times with 0.1 M PHEM buffer and embedded in 12% gelatin in 0.1 M PHEM buffer at 37°C. Cells were centrifuged for two minutes at 15000 rpm and gelatin was solidified on ice. 1 mm^3 cubes were cut, which were impregnated overnight on a turning wheel with 2.3 M sucrose at 4°C (13). With a cryoultramicrotome (UC6 FC6, Leica, Microsystems, Vienna, Austria), 50-55 nm sections were cut for electron microscopy. Sections were picked up with 1.15 M sucrose/0.1% methylcellulose

in 0.05M PHEM buffer and dipped on formvar/carbon coated Cu grids (Stork Veco B.V., Eerbeek, the Netherlands) for electron microscopy.

Mounted sections of JBY20 were washed five times with PBS, pH 7.4 137 mM NaCl, 8 mM Na_2HPO_4, 2.7 mM KCl, 1.5 mM KH_2PO_4)/ 0.02 M Glycine. The sections were blocked by one incubation in PBS, 1% BSA (Sigma, Zwijndrecht, the Netherlands) and subsequently incubated with mouse 12CA5 anti-HA antibody (Roche Diagnostics) diluted 1:300 in PBS, 1% BSA for one hour. The labeled sections were washed 5 times 2 minutes with PBS, 0.1% BSA and incubated for 20 minutes with rabbit-anti-mouse IgG (Dako Denmark A/S, Glostrup, Denmark) diluted 1:250 in PBS, 1% BSA. The sections were washed five times two minutes with PBS, 0.1% BSA and then incubated 20 minutes with protein A 10 nm gold (Dept. Cell Biology, University Medical Centre Utrecht, the Netherlands) diluted 1:90 in PBS, 1% BSA. The sections were then rinsed three times shortly and seven times for two minutes with PBS. The labeled sections were fixed for 5 minutes in PBS, 1% glutaraldehyde and subsequently washed ten times with aqua dest. This was followed by an incubation of 5 minutes in uranyl oxalate, pH 7.4 (14) and two washes with aqua dest followed. Two rinses on ice on 0.4% aequous uranyl acetate/methylcellulose, pH 4.5 followed and the incubation was continued with fresh solution for 5 minutes on ice. Excess of fluid was then drained off and the sections were dried at room temperature.

The sections were observed with a JEOL 1010 transmission electron microscope (JEOL, Tachikawa, Japan) at 80 kV and images were recorded on Agfa Scientia sheet film. The scanned pictures were processed with Photoshop 5.5.

Immunoprecipitation

Samples were washed once with ice-cold H_2O and cells were collected by centrifugation. Cells were suspended in 500 µl Tris/HCl pH 7.4 supplemented with complete protease inhibitors (Roche Diagnostics), sodium fluoride and benzamidine, and subsequently the cells were lysed by vigorously shaking with 0.45 mm glass beads in a Mini BeadBeater 8 (Glen Mills Inc.,

New Jersey, USA) for 1.5 min. 500 µl Tris/HCl pH 7.4 containing protease inhibitors, sodium fluoride, benzamidine and 1% Triton X-100 was added, the sample was mixed and 800 µl lysate was collected. Hxt5-HA was immunoprecipitated from pre-cleared lysates with the use of 12CA5 anti-HA antibody (Roche Diagnostics) coupled to protein A or G sepharose beads (Sigma), respectively. After binding, the beads were washed four times with Tris/HCl pH 7.4, sodium fluoride, benzamidin and 0.5% Triton X-100. 1.2x reducing sample buffer was added and immunoprecipitates were separated on SDS-PAGE gel. The proteins were transferred to PVDF membrane and detected with Fk2 anti-ubiquitin (Biomol, Exeter, United Kingdom) or anti-phosphoSerine (Calbiochem, EMD Biosciences, Inc., San Diego, CA, USA) and rabbit-anti-mouse peroxidase (Jackson Immunoresearch) and visualized with Enhanced Chemiluminescence (Renaissance).

Results

Induction of Hxt5p degradation.

Hxt5p expression is known to be induced when cells have a low growth rate and repressed when the growth rate is increased. Within one hour after transfer of stationary phase cells to fresh, 2% glucose containing YNB medium, *HXT5* mRNA is not detected anymore (8). *HXT5* tagged in the genome with an *HA*-sequence was used to determine the rate of Hxt5 protein removal from the culture after transfer of stationary phase cells to fresh, 2% glucose containing medium. We chose to use tagged Hxt5 for our experiments as there are no antibodies available for native Hxt5 protein. As shown in Fig. 1A, stationary phase cells express Hxt5-HA. Addition of glucose to the cells resulted in a decrease of the Hxt5-HA level to 66% of the initial level within one hour (Fig. 1A/C). Interestingly, the cells were still in the lag phase during this period, indicating that the induction of Hxt5-HA expression was inhibited and the degradation was induced immediately after the addition of glucose (Fig. 1B). While the OD_{600} of the culture started to increase between one and two hours after the addition of glucose, the amount of Hxt5-HA decreased further to 59% of the initial amount of Hxt5-HA after 2 hours and 21% after 4 hours when the

culture reached the exponential phase of batch growth (Fig. 1). Thus while *HXT5* is not expressed anymore one hour after stationary phase cells were transferred to growth rate promoting medium, the Hxt5-HA protein was more slowly degraded and there was still Hxt5-HA protein present four hours after cells were transferred to fresh medium. The decrease in Hxt5-HA protein levels coincided with an increase in the growth rate, in agreement with previous results (8).The fact that the initial decrease of the amount of Hxt5-HA occurred already during the lag phase of the cells in fresh medium suggests for an active degradation process of Hxt5p, which was initiated by the cell prior to the increase in the growth rate of the culture.

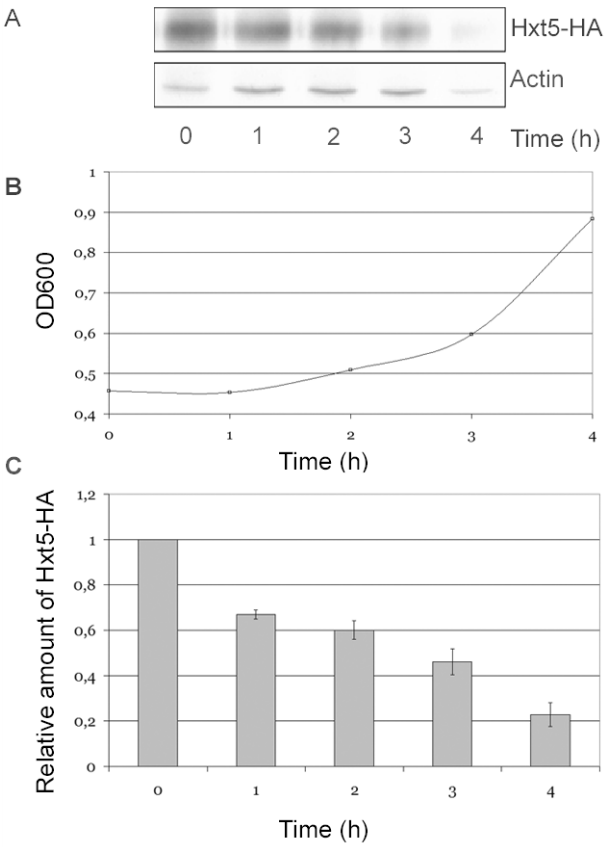

Figure 1: **Hxt5-HA protein levels in starved cells and after induction of the growth rate.** Strain JBY20 was grown to stationary phase and transferred to fresh, 2% glucose containing YNB medium. Cells were collected at the moment of inoculation and at each hour. (A) Western blot analysis of Hxt5-HA. (B) Optical density at 600nm. (C) Quantification of Hxt5-HA levels, error bars indicate the standard error of the mean (n=5).

Hxt5-HA degradation

It has been known for a long time that proteins destined for rapid degradation contain a so-called PEST sequence. In *Saccharomyces cerevisiae* ubiquitination of other transporters such as the galactose transporter and the maltose transporter is reported also to be dependent on a PEST sequence (15, 16). Database research and algorithm analysis (http://emb1.bcc.univie.ac.at/embnet/tools/bio/PESTfind) on Hxt5p indicated a PEST-region located at amino acids 302-325, which is in the longest intracellular part of the protein. PEST elements often contain phosphorylation sites that play a role in targeting proteins for degradation (17). To determine whether phosphorylation of one or more serine residues coincided with Hxt5-HA degradation, the following immunoprecipitation experiment was performed. Cells were grown to the stationary phase (t=0min) and transferred to fresh, 2% glucose containing medium. At different time points Hxt5-HA was immunoprecipitated and after gelelectrophoresis and Western blotting detected with anti-phosphoserine antibodies. As shown in Fig. 2B, the precipated Hxt5-HA clearly exhibited anti-phosphoserine reactivity, which increased transiently after the shift of the cells to glucose-containing medium. The maximal reactivity was obtained 10 – 30 minutes after the medium shift, and reactivity returned to basal levels 60 minutes after the medium change (fig. 2).

In *S. cerevisiae*, it is known that not only cytosolic proteins that are selected for degradation in the proteasome can be covalently linked to ubiquitin, but also transmembrane proteins that are degraded via the endocytic pathway may have ubiquitin attached. Examples of these transmembrane proteins are the galactose transporter Gal2 (15) and the maltose permease (16). The PEST sequence of these proteins has been shown to be involved in the ubiquitination of these transporters. To investigate whether Hxt5-HA becomes ubiquitinated, stationary phase cells and stationary phase cells incubated in fresh, 2% glucose containing YNB medium for 10, 30 and 60 minutes were subjected to immunoprecipitation using anti-HA antibodies to precipitate Hxt5-HA. After gel-electrophoresis and Western

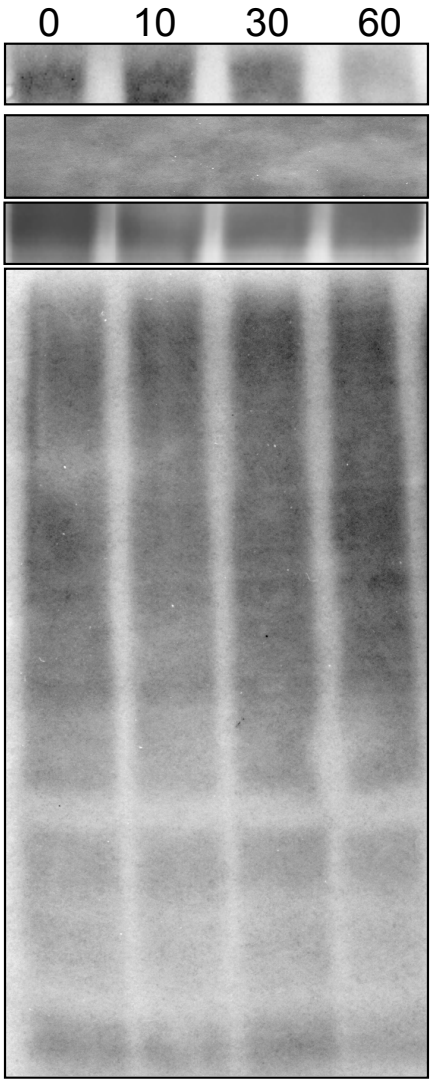

Figure 2: ***In vivo* ubiquitination and phosphorylation of Hxt5-HA.** Strain JBY20 was grown to starvation and transferred to fresh, 2% glucose containing medium. Cells were collected at the moment of inoculation into fresh medium and at the time points indicated and subsequently Hxt5-HA was immunoprecipitated. After electrophoresis and Western blotting, the membranes were detected for ubiquitination (A) and phosporylation of serine residues (B) of Hxt5-HA. The immunoprecipitated Hxt5-HA was detected and presented in panel C. Whole cell lysates were detected for ubiquitinated proteins and presented in panel D.

blotting, the blots were detected with anti-ubiquitin or anti-HA as indicated (Fig. 2A). Although Hxt5-HA was present in the immunoprecipitates (Fig. 2C), no anti-ubiquitin signal was detected (Fig. 2A). The fact that no ubiquitination of Hxt5p could be found was not due to the experimental conditions, as the cell lysates obtained in this experiment clearly exhibited anti-ubiquitin reactivity (Fig. 2D). These observations suggest that Hxt5-HA is not ubiquitinated or only to a low level. Together, these results demonstrate that exposure of stationary phase cells to glucose results in rapid, transient phosphorylation of

Hxt5-HA, but not in ubiquitination of the protein. Therefore, the involvement of the PEST sequence in the degradation of Hxt5-HA is unlikely.

Hxt5-HA localization

In order to elaborate on the site of Hxt5-HA degradation, the localization of the protein has been studied using immunofluorescence microscopy. Stationary phase cells were treated, and subsequently subjected to immunofluorescence microscopy as described under Materials and Methods. As shown in Fig. 3, Hxt5-HA labelling in stationary phase cells exhibited a localization at the cell periphery (Fig. 3, t=0h), which indicates that the transmembrane protein Hxt5-HA is localized at the plasma membrane during stationary phase.

During the first two hours after transfer of cells to fresh, 2% glucose containing medium, the localization of Hxt5-HA changed from a predominant membrane localization to both membrane localization and some cytoplasmic staining, the latter as a punctuate structure, while the intensity of the signal of single cells decreased (Fig. 3A/B, t=0h, t=2h, filled arrowheads). The decrease in labelling intensity explained partly the decrease in signal observed on the Western blot between t=0h and t=2h. Another reason for the decrease in this signal is that in contrast to stationary phase cells, two hours after transfer not all the cells exhibited Hxt5-HA label anymore (Fig. 3A/B, filled arrows). More cells lacked Hxt5-HA labelling at t=3h and t=4h in comparison to cells in the stationary phase (filled arrows), where all cells were labelled. At t=4h, the membrane labelling was faint and almost absent; the label present was more internalized than during earlier time points During stationary phase, some of the cells are able to progress slowly through the cell cycle. The buds of these cells were labelled for Hxt5-HA (Fig. 3A/B, t=0, open arrowhead), but two hours after transfer of the cells to fresh medium, new (developing) daughter cells no longer exhibited Hxt5-HA labelling (Fig. 3A/B, t=2, t=4, double-headed arrows). The internalization and subsequent degradation of Hxt5-HA was therefore not the only cause of the decrease in signal in figure 1, but the decrease in Hxt5-HA was also caused by prevention of Hxt5-HA to be

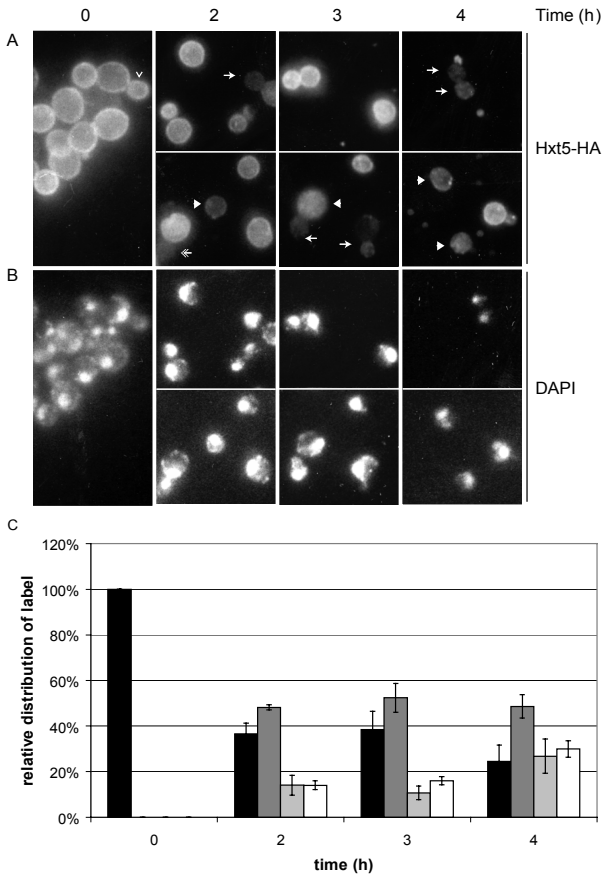

Figure 3. **Immunofluorescence of Hxt5-HA in starved cells and after induction of growth.** Strain JBY20 was grown to stationary phase and transferred to fresh, 2% glucose containing YNB medium. Cells were collected at the moment of inoculation into fresh medium and at each hour. (A) Hxt5-HA labelling of whole fixed cells. (B) Nuclear staining with DAPI of whole, fixed cells. Quantification of the immunofluorescence is shown in panel C, with cells showing strong peripheral labelling (black bars), a weak peripheral labelling (dark grey bars), punctate internal labelling (light grey bars) and no labelling (white bars).

incorporated into new buds, because *HXT5::HA* was no longer expressed and the existing proteins were not able to diffuse into the membrane of the developing bud.

Quantification of the immunofluorescence pictures revealed that two hours after increase of the growth rate, the percentage of cells with strong

peripheral labelling had decreased from 100% to 27% (Fig. 3C). This percentage was roughly the same at three hours after induction of the growth rate, but when the cells reached the exponential phase, this percentage has dropped to 24%. The percentage of cells that contained a weak peripheral labelling increased from 0% at t=0h, to 48% after two hours. This labelling was also relatively the same at three hours after increase of the growth rate, and remained constant for the next hours. It seemed that the increase in weak peripheral labelling arose from cells that had lost their strong membrane labelling. Besides cells with a weak overall labelling, cells without label at the periphery but with punctate labelling in the cell interior appeared. Two hours after stationary phase cells were transferred to fresh, 2% glucose containing medium, 13% of the cells showed punctate labelling, and this increased to 17% at three hours after transfer. When the cells reached the exponential phase of batch growth at four hours after transfer, this percentage increased rapidly to 40%.

In addition, unlabeled cells appeared. These cells are most likely the daughter cells of mothers, which did not incorporate Hxt5-HA in the buds anymore when they started budding after the addition of growth rate promoting medium. The percentage of unlabeled cells was relatively constant from two to three hours after the cells were transferred to growth rate promoting medium, but increased in the following hour when the cells entered the exponential phase of batch growth.

Taken together, these data suggest that the decrease in signal observed on Western blot is caused by both dilution of Hxt5-HA proteins out of the culture and by internalization and degradation.

Ultrastructural localization of Hxt5-HA

Immunofluorescence microscopy allowed us to define the localization of Hxt5-HA described above to either the cell periphery or intracellular sites, but to examine the internalization and degradation of Hxt5-HA in more detail, we used stationary phase cells and performed immunogold labelling on cryosections prepared according to Tokuyasu (13). Figure 4 shows a labelling

of Hxt5-HA at the plasma membrane (PM) in stationary phase cells. The label present in the interior of the cell is partially localized to the endoplasmic reticulum (ER, arrow), vesicular structures (VS) and the vacuole (V). This labelling suggests a turnover of Hxt5 protein in the stationary phase. The ER location is for newly synthesized Hxt5p, while the localization to vesicles and the vacuole stands for the degradation pathway. Very little labelling was present in the nucleus (N) or mitochondria (M). This internal labelling is in analogy with the published degradation route of Hxt2p and Hxt7p, which are internalized via the endocytic pathway (18-20).

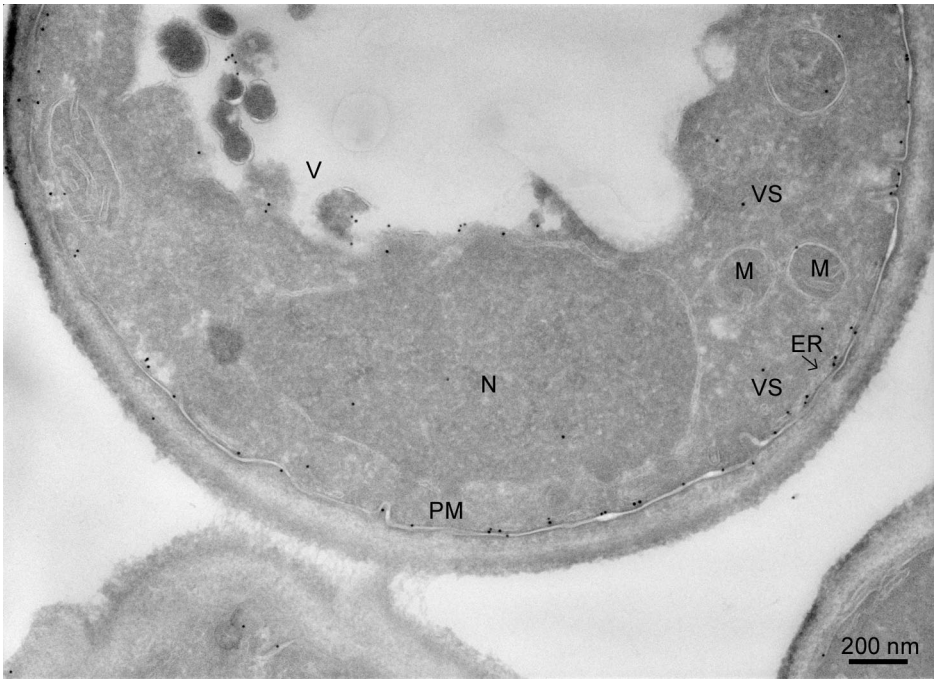

Figure 4. **Immunogoldlabeling on cryosections of Hxt5-HA in starved cells.** Strain JBY20 was grown to stationary phase and transferred to fresh, 2% glucose containing YNB medium. Cells were collected 48 hours after the inoculation of batch growth, , and Hxt5-HA was labeled with immunogold for electron microscopy. Several ultrastructures are indicated in the figure: CM, cell membrane; N, nucleus; M, mitochondrion; V, vacuole; VS, vesicular structure; ER, endoplasmic reticulum. Scale bar 200 nm.

Discussion

The expression of *HXT5-HA* is regulated by the growth rate and not by the extracellular glucose concentration as is the case for the major hexose transporters (2, 5, 8, 9, 21-26).*HXT5* is expressed at low growth rates in nitrogen-limited cultures irrespective of the high glucose concentration (200 mM) in the medium (8). The regulation of *HXT5* expression is controlled by STRE and HAP elements in the promoter (10). Although HAP elements are regulated by glucose (27, 28), they are independent of Hxk2p. Hxk2p influences the expression of the major hexose transporters dependent on the extracellular glucose concentration (2, 11, 29), but deletion of *HXK2* did not derepress the expression of *HXT5* (11). The molecular structure of Hxt5-HA on the other hand is quite similar to that of the major hexose transporters, only the amino-terminal domain of Hxt5-HA differs significantly from that of the major hexose transporters (8). Because the induction of *HXT5* expression is regulated differently than that of the major hexose transporters, we were interested whether the metabolism of the removal of Hxt5-HA from the culture was also regulated differently as compared to the major hexose transporters (18-20, 24). Biochemical and immunofluorescence studies demonstrated that endocytosis, autophagy, and transport to the vacuole are involved in degradation of the major hexose transporters Hxt2p, Hxt6p and Hxt7p (18-20, 24). Krampe et al. (19) reported a possible involvement of ubiquitination in the degradation of Hxt6p and Hxt7p. No data are available for ubiquitination of the other major hexose transporters.

Hxt5 protein levels on Western blot decreased when cells were transferred to growth-promoting medium. Immunofluorescence studies revealed that Hxt5-HA translocated from the cell periphery to cell interior, which suggested internalization and degradation. Stationary phase cells demonstrated a cell periphery labelling. More internalized label and more unlabeled cells were visible when cells were kept longer on growth-rate-promoting medium. This suggests an active degradation process for Hxt5p. Hxt5-HA was also diluted out of the culture because mother cells in fresh, 2% glucose did not incorporate Hxt5-HA in the developing bud, while they did

during the stationary phase. The increase in internal labelling showed that the degradation of Hxt5-HA was induced by transfer to growth rate promoting medium, because the internal labelling would otherwise not have increased from t=0h to t=4h. The decrease in Hxt5p signal on Western blot is thus caused by both degradation and dilution of Hxt5p out of the culture.

The clear vacuolar degradation that was observed in previous studies of Hxt7p (20, 24), which showed a sole vacuole label of Hxt7-GFP, was not found for Hxt5-HA. The vacuolar degradation of Hxt7 was only observed eight hours after transferring the cells to medium in which *HXT7* is no longer expressed; Eight hours after *HXT5-HA* expressing cells were transferred to fresh medium, the growth rate became slower again as the glucose concentration became low. The cells were approaching stationary phase again and therefore *HXT5* expression was again induced.

For Hxt7p, it has been show that the amino- and carboxyterminal domains of the protein are necessary for degradation (20). A part of the amino-terminal domain of Hxt7p is described to be enriched in proline, glutamic acid, serine and threonine amino acids (PEST-sequence like) and such sequences are implicated in ubiquitin-dependent proteolysis (17, 20). Phosphorylation of serine residues in the PEST-like sequence of the membrane protein uracil permease was shown to be involved in ubiquitination and subsequent vacuolar degradation of this transporter (30). Database research clearly demonstrated the presence of a PEST-sequence in the middle part of the Hxt5 protein, which is in the longest intracellular loop between transmembrane domains 6 and 7. PEST sequences have been demonstrated before in other permeases such as the maltose permease and the uracil permease (16, 30). Immunodetection with anti-phospho-serine antibodies demonstrated phosphorylation of at least one serine residue of Hxt5-HA. This was in analogy with data for the uracil permease (*FYR4* gene), of which multiple serine residues of the PEST-sequence become phosphorylated before degradation (30). A PEST-like sequence has been reported also for Hxt7 and it is known that the ubiquitin system is involved in the degradation of this transporter (20). However, no ubiquitination was found in immunoprecipitated Hxt5-HA by detection with anti-ubiquitin antibodies.

Therefore, we conclude that the PEST-sequence is probably not involved in the degradation of Hxt5-HA. The phosphorylation of Hxt5-HA might instead indicate a regulatory signal induced by improved environmental conditions, because Hxt5-HA levels only decreased to 66% during the time of Hxt5-HA phosphorylation on one or more serine residues.

Labelling of cryosections was used to examine in more detail the localization of Hxt5 in stationary phase cells. The ultrastructural details were well preserved and several organelles, such as nucleus, mitochondria and plasma membrane, were identified. In cryosections of stationary phase cells labelled for electron microscopy, the plasma membrane was labelled. The internal ultrastructures that labelled during the stationary phase were the vacuole and probably endocytic structures. The labelling of Hxt5-HA in the ER, endocytic structures and the vacuole is most likely due to constitutive renewal of Hxt5-HA.

In conclusion, these data indicate that Hxt5p is degraded via the same mechanisms as the major hexose transporters Hxt2p, Hxt6p and Hxt7p and other transporters, namely via endocytosis and transport to plus degradation in the vacuole. Thus, while the expression of *HXT5* and the regulation of degradation of Hxt5-HA are regulated differently than that of the major hexose transporters, the degradation mechanisms are similar.

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Chapter 7

General Discussion

Saccharomyces cerevisiae is able to adjust the length of the cell cycle by regulation of the length of the G1 phase (1, 2). Several extracellular variables are able to modulate the length of the G1 phase and one important factor is nutrient supply. The length of the G1 phase slowly increases with decreasing fluxes of galactose until a switch point is reached; below that point the G1 phase is swiftly elongated (3). In Chapter 3 is described that not only below a galactose flux of 18 fmol cell-1 h-1, the G1 phase rapidly elongates, but also below a glucose flux of 14 fmol cell-1 h-1. Elongation of the G1 phase is known to be accompanied by accumulation of the reserve carbohydrates trehalose and glycogen (3, 4). The amount of energy used to accumulate the reserve carbohydrates is not available for cell cycle progression, which results in an elongated G1 phase. Cells that are deleted for the ability to store both trehalose and glycogen are also unable to elongate their G1 phase; probably they use all available energy for cell cycle progression. These results demonstrate that the accumulation of trehalose and glycogen is thus not caused by the elongation of the G1 phase, but that the elongation of the G1 phase is caused by the accumulation of the reserve carbohydrates trehalose and glycogen.

CLN3 is the first cyclin to be expressed in the G1 phase and Cln3p forms a complex with the cyclin-dependent kinase Cdc28 (5, 6). The Cln3-Cdc28 complex induces transcription of the other two G1 cyclins, *CLN1* and *CLN2*, which encode highly homologous proteins (7-9). The transcription of *CLN1* and *CLN2* is facilitated by the SBF transcription factor complex, which consists of the nuclear, DNA binding protein Swi4 and the regulatory component Swi6p, which is nuclear during the G1 phase and cytosolic during the S, G2 and M phase (10-13). The localization of Swi6p is regulated by phosphorylation on serine 160 by Slt2p (11). Slt2p is the MAPK of the Pkc1-Slt2 cell wall integrity pathway and this pathway can be activated by membrane receptors or Cdc28p; although the corresponding cyclin for Cdc28 is not determined yet, it was suggested that Cln1p or Cln2p is this activating cyclin (14-21). Cln1 protein levels were to dependent on the length of the G1 phase and on the source, while Cln3 protein expression patterns were not influenced by these variables. Cln1p might therefore be used to determine the position in the cell cycle while Cln3p is not suitable for that purpose.

Swi4 protein expression was restricted to the G1 phase in cells with a short G1 phase and undetectable in cells with an elongated G1 phase, which might indicate that the Swi4p levels were lower during an elongated G1 phase. However, the reduced synchrony of cultures with elongated G1 phases might decrease the amount of Swi4 per time point below detection level, while the total amount of Swi4p per cell is not altered. Swi6 protein patterns were dependent on the carbon source provided to the cells. During both short and elongated G1 phases on glucose, Swi6 protein was present throughout the cell cycle.Swi6p levels disappeared after entry into S phase during both short and elongated G1 phases on galactose. The phosphorylation of Swi6p appeared at the end of the G1 phase in agreement by the study of Sidorova et al. (11).

The activity of the MAPK Slt2p preceded the phosphorylation of Swi6p and continued as long as phosphorylated Swi6p was present. Deletion of the *SLT2* gene resulted in severe elongation of the cell cycle. Although more research is needed, the increase in percentage of budded *slt2*Δ cells compared to the percentage of budded wild type cells during batch growth, suggests that *slt2*Δ cells are restricted at the morphogenesis checkpoint, which is located in the G2 phase. Only Swi4p is required for the expression of cell wall genes (22). Therefore, it is suggested that the phosphorylation of Swi6p enables a cell to not only express the cyclins required for cell cycle progression via the SBF complex, but to also express cell wall genes needed to modulate and expand the cell wall. This cell wall modelling is necessary for bud formation and bud growth. In this way, the cell can coordinate DNA replication and cell morphology alteration during the cell cycle.

The regulated activity of another MAPK has been shown before in yeast cells for the pheromone MAPK pathway but activity of this pathway arrests a cell in the G1 phase (23). This indicates that activity of MAPK signal transduction pathways is not always favourable for cell cycle progression. MAPK signal transduction pathways are preserved from yeast to mammals and MAPK activity is also regulated in mammalian and other animal cells during the cell cycle (24). The best-studied MAPK pathway leads to activation of $p44^{MAPK}$ and $p42^{MAPK}$ and the activity of this cascade is involved in cell cycle

progression during all phases of the cell cycle (25-31). The involvement of yeast MAPK cascades in cell cycle progression is also not restricted to the G1 phase. The homology between yeast and mammalian MAPK pathways is demonstrated in the processes in which the pathways of the different species are involved. During the G1 phase, the yeast Pkc1-Slt2 pathway is involved, but also the mammalian MAPK cascade plays a role in progression through the G1 phase. Both pathways are also involved during M phase in spindle pole body formation or monitoring of its status (22, 26-28).

Quiescent mammalian cells are arrested in the G1 phase and reactivation of these cells is associated with MAPK activity (29). MAPK activity is also linked to the G1/S transition (26, 30, 31). The level of MAPK activity must be controlled as well, as both too high and too low activity of the MAPK pathway lead to cell cycle arrest (24, 32, 33). DNA synthesis and cell proliferation is blocked in mammalian cells when the activity of the MAPK pathway is inhibited by antisense constructs, overexpression of kinase inactive mutant proteins or inactivation by MPK-1 (30, 31). In *S. cerevisiae*, inhibition of Slt2p activity also results in severe cell cycle elongation. Although no data are published about the direct link between increased Slt2p activity and cell cycle progression, elevated Slt2 activity after stress is also known to correlate with cell cycle delay. *E.g*. mild heat shock and osmotic shock are both known to delay both bud formation and nuclear division and both stresses are demonstrated to increase Slt2p activity (34-37). Thus, the regulation of cell cycle progression by MAPK seems to be conserved from yeast to mammals. Not only is the activity of MAPK required for cell cycle progression in both yeast and mammals, overactivity of MAPK results in cell cycle delay.

The prolonged Slt2p activity in a cell cycle with an elongated G1 phase might also be caused by the accumulation of trehalose. The increased osmolarity that is caused by accumulation of trehalose activates the HOG pathway. This leads to activation of the Pkc1-Slt2 pathway in order to remodel the cell wall (38). Further research is required to explore the activity of Slt2p in mutants unable to store trehalose, glycogen or both. With that research, it should be possible to elucidate whether the Slt2 activation, during G1 phase

and after S phase entry, is an affect of trehalose accumulation in elongated cell cycles.

Elongation of the G1 phase was demonstrated before in Chapter 3 to be regulated by the accumulation of the reserve carbohydrates trehalose and glycogen. The protein expression of Tps1 did not differ between short and elongated G1 phases (data not shown) and therefore it is likely that the activity of the trehalose accumulating complex is regulated. It has been suggested before that Tps1p, or its product trehalose-6-phosphate, regulates the import of glucose into the cell by regulation of the Hxt proteins, or the influx of glucose into glycolysis. Because Tps1p is required during all stages of batch growth on glucose, an additional regulatory mechanism should restrict trehalose accumulation to the diauxic shift and the stationary phase. In Chapter 4, the mutual influence between Tps1/trehalose accumulation and the hexose transporters *HXT1/7* was investigated. Our data show that *TPS1* and Tps1p are expressed throughout batch culture on glucose with an increase in mRNA expression around the diauxic shift when trehalose accumulation was initiated Although the expression of the hexose transporters was regulated and the expression of individual hexose transporters was restricted to specific periods of batch growth on glucose, the expression of *TPS1* or trehalose accumulation did not coincide with one hexose transporter specifically. During the stationary phase however, only *HXT5* was expressed while trehalose accumulation continued. Trehalose accumulated at that time had to result from gluconeogenesis as glucose in the medium was exhausted by then. Single deletion of *HXT-/5* or double deletion of *HXT6/7* also had no clear effect on the accumulation of trehalose after 24 hours of batch growth, although the lack of effect could well result of redundancy mechanisms as trehalose accumulation is very important for cell viability (39-43). Trehalose accumulation is related to low growth rates (3, 4, 42) and stress conditions (39-47) and we have previously published that expression of *HXT5* is also regulated by the growth rate (48). Fed-batch and continuous culturing data presented in Chapter 4 indicate that trehalose accumulation coincided with *HXT5* expression under all conditions tested. The correlation between *HXT5* expression and trehalose accumulation was strengthened by

coimmunoprecipitation of Hxt5-GFP with Tps1p. Addition of glucose to wild-type stationary phase cells, resulted in coimmunoprecipitation of Tps1p and Hxt5p. This binding was lost after 30 minutes, indicating that Tps1p only transiently binds Hxt5p after addition of glucose, or that after addition of glucose the Tps1-Hxt5 complex is more stable. We propose the existence of a functional complex of Hxt5p and Tps1p during stationary phase. In this complex, probably Hxk2p is included, because the early steps in glucose metabolism can then be exerted in a vectorial process. In addition, *tps1*Δ cells become able to grow on glucose upon deletion of *HXK2* (49) and this supports that some interaction exists between Hxk2p and Tps1p. In the suggested model, glucose transported into the cell by Hxt5p is phosphorylated by Hxk2p and subsequently shuttled to trehalose accumulation via Tps1 or to the glycolysis. This complex would enable a cell to cope with fresh glucose after exhaustion and therefore we named it the glucose-response complex (GRC).

As Hxt5p was suggested to be a hexose transporter which would transport glucose after this became available again (50), Hxt5p was expected to localize at the plasma membrane. Electron microscopy studies showed however that Hxt5p was localized mainly at the endoplasmic reticulum and hardly at the plasma membrane. This study again shows the great value of high resolution images obtained by election microscopy as Hxt5p was initially localized to the plasma membrane by (immuno)fluorescence examination of the localization of Hxt5-GFP or Hxt5-HA. The part of Hxt5p that is localized to the plasma membrane might account for the initial glucose uptake once this carbon source becomes available again. Although the Gal2p galactose transporter is also thought to be able to transport glucose (51), initial influx of glucose is probably completely dependent on Hxt5p activity during growth on non-fermentable carbon sources like ethanol. The low presence of Hxt5p on the plasma membrane might explain the low growth rate of an *HXT5*-only mutant while the affinity of this transporter for glucose is moderate (50).

A possible role for Hxt5p in the endoplasmic reticulum membrane might be found by homology of Hxt5p and GLUT7 in mammalian cells (52). GLUT7 is also localized at the endoplasmic reticulum and is responsible for transport of glucose derived by gluconeogenesis out of the endoplasmic

reticulum. The final step of gluconeogenesis, from glucose-6-phosphate to glucose, is in mammalian cells performed in the endoplasmic reticulum. In yeast cells, the localization of gluconeogenesis is not known, but might be exerted at the same place as in mammalian cells. The localization of Hxt5p and the correlation between *HXT5* expression and trehalose accumulation strengthen the suggestion that the glucose stored as trehalose after the diauxic shift is derived from gluconeogenesis and that Hxt5p is involved in these processes.

Although is seems not logical for yeast cells to make free glucose, which can be transported out of the cells via hexose transporters, it was reported before that Hxt5p plays a role in efflux of glucose during ethanol-limited continuous cultures (53). The low abundance of Hxt5p on the plasma membrane compared to amount of Hxt5p on the ER membranes might restrict this efflux of glucose. The glucose obtained by gluconeogenesis might be immediately converted to trehalose via the TPS complex which is bound to Hxt5p. The minor loss of glucose via Hxt5p in the plasma membrane might be the offer yeast cells bring to be also able to respond quickly to new glucose.

The mechanism by which the GRC regulates the influx of glucose into glycoslysis was studied in Chapter 5. Disruption of the GRC by deletion of *TPS1* had a strong effect on the expression of all of the hexose transporters. Expression of the hexose transporters was not induced when *tps1*Δ cells were transferred to glucose containing medium. Although these data might suggest a stimulatory role for *TPS1* in the expression of the hexose transporters, it is more likely that rapid energy depletion after glucose addition was responsible. This assumption is strengthened by the report of Thevelein and Hohmann (54) that addition of glucose, even at very low concentrations, to galactose grown *tps1*Δ caused growth arrest by rapid depletion of ATP and P_i, together with an increase in initial sugar phosphates of glycolysis, especially fructose-1,6-biphosphate (54).

In Chapter 6, the metabolism of Hxt5p was studied. Hxt5p was removed from the cultures by both degradation and diminishing the number of cells containing Hxt5p. The lowering of Hxt5p positive cells was achieved by

restriction of Hxt5p to the mother cells and no incorporation of Hxt5p into daughter cells anymore. Ubiquitination of Hxt5p was not detected, although the protein sequence contains a putative PEST sequence and the Hxt5 protein was transiently phosphorylated on one or more serine residues after the addition of glucose to stationary phase cells. This serine phosphorylation coincided with the interaction of Tps1p and Hxt5p, and therefore this serine phosphorylation might not be a degradation signal but an activity regulating signal. Another clue for this assumption is that the transient phosphorylation was gone within one hour, whereas more than 60% of the protein was still present at that time. The phosphorylation of Hxt5p further coincided with the coimmunoprecipitation of Hxt5p and Tps1p. This indicates that the phosphorylation of Hxt5p and the activity of the GRC are linked.

Electron microscopic analysis showed that the majority of Hxt5p was localized to the endoplasmic reticulum (ER) just below the plasma membrane and only a small fraction was localized to the plasma membrane itself (see Chapter 4). Examination of the localization of Hxt5p in stationary phase cells revealed that Hxt5p was not only localized at the endoplasmic reticulum and the plasma membrane, but also at vesicular structures and the vacuole. A part of the labelling at the endoplasmic reticulum and the labelling in vesicular structures and the vacuole might represent the renewal of Hxt5p at the plasma membrane. This degradation pathway for Hxt5p is similar to that of the major hexose transporters Hxt2p, Hxt6p and Hxt7p. Together, the data presented in Chapter 4, 5 and 6 make clear that Hxt5p has very different roles in growth than the major hexose transporters and is subject to modification by phosphorylation, but is degraded in via similar routes.

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Samenvatting

De gist *Saccharomyces cerevisiae* is bij de meeste mensen bekend onder de naam 'bakkersgist'. De bekendste toepassing van deze gist is het gebruik voor het rijzen van brood, maar gist wordt ook gebruikt voor de productie van alcohol in bier en wijn.

Gisten, maar ook planten, mensen en dieren zijn eukaryote organismen terwijl bacteriën prokaryoot zijn. Het verschil tussen deze twee groepen is dat in prokaryoten alle processen in de cel in één ruimte plaatsvinden, en in eukaryoten is de cel opgedeeld in 'hokjes', de zogenaamde organellen. In deze organellen worden verschillende processen in de cel gescheiden en gereguleerd. Voorbeelden van die organellen zijn de kern en het mitochondrion. In de kern ligt erfelijk materiaal (DNA) opgeslagen en een mitochondrion is vergelijkbaar met een energiecentrale van een cel. Het DNA in een kern bevat heel veel informatie. Deze informatie is opgedeeld in kleinere stukjes, zoals een hoofdstukje in een encyclopedie. Elk hoofdstukje heet een gen, en een cel kan dit hoofdstukje lezen en vertalen naar een boodschapper RNA of messenger RNA (mRNA). Hoe vaak een cel dit hoofdstukje leest, dus hoeveel mRNA er gemaakt wordt, is een maat voor de expressie van een gen. Deze expressie wordt gereguleerd door een stukje DNA net voor het gen (de promoter) samen met eiwitten die aan deze promoter binden. Het gemaakte mRNA wordt buiten de kern, in het organel 'endoplasmatisch reticulum', vertaald in eiwit.

Gist is een organisme dat bestaat uit één cel, terwijl mensen en dieren uit miljarden cellen bestaan en daardoor multi-cellulaire organismes zijn. Door hun ééncelligheid worden gisten vaak gebruikt als modelsysteem om processen in menselijke of dierlijke cellen mee te bestuderen. De gistcel is in staat zich veel sneller voort te planten dan mensen en dieren als organismen en sneller dan menselijke en dierlijke cellen in laboratoriumkweek. Eén van de processen die bestudeerd wordt in gist is dan ook de celdeling. De celdeling wordt ook wel celcyclus genoemd en wordt opgedeeld in vier fasen. (Hfdst. 2, fig. 1) namelijk de G1, S, G2 en M fase. In de G1 fase bereidt een cel zich voor op de komende celcyclus. In de S fase wordt het DNA verdubbeld. In de G2 fase

wordt onder andere gecontroleerd of het verdubbelen van het DNA goed is verlopen en of de structuur van de cel het toestaat om de gaan delen. In de M fase wordt het verdubbelde DNA netjes verdeeld over de twee nieuwe cellen en splitsen de cellen zich van elkaar.

Om ervoor te zorgen, dat deze complexe processen goed verlopen, hebben eukaryote cellen controlepunten ingebouwd in hun celcyclus. Wanneer er ernstige fouten ontstaan in deze controlepunten, kan dat ertoe leiden dat een cel niet meer deelt, of ongecontroleerd gaat delen. In multi-cellulaire organismes is deze laatste verstoring bekend als kanker. De te controleren processen in de G2 fase zijn hierboven genoemd. In de G1 fase controleert een cel of de omgeving goed genoeg is om een nieuwe ronde van de celcyclus te starten. In gist is er één controlepunt in de G1 fase en dit punt heet 'Start'. Eén van de belangrijkste omgevingsfactoren die de cel meet op Start is de hoeveelheid nutriënten. De hoeveelheid nutriënten regelt hoe snel een cel de G1 fase kan doorlopen. Ook de kwaliteit van de nutriënten speelt een rol in de lengte van de G1 fase. Cellen halen de meeste energie uit glucose en groeien daarom ook het snelst op glucose. Omdat de lengte van de celcyclus voornamelijk bepaald wordt door de lengte van de G1 fase, is de G1 fase op glucose dus het kortst. De aanwezigheid van nutriënten regelt de snelheid en hoeveelheid van de aanmaak van bepaalde eiwitten de belangrijk zijn voor de progressie door de celcyclus. Deze eiwitten zijn specifiek voor de fase van de celcyclus en heten cyclines. Er zijn drie G1-fase cyclines en deze activeren om beurten het eiwit Cdc28 tijdens de G1 fase. Cln3 is het eerste eiwit dat verhoogd wordt in de G1 fase en het Cln3-Cd28 complex zorgt voor vorming van een complex van Swi4 en Swi6. Dit Swi4,6 complex in betrokken bij de transcriptie van *CLN1* en *CLN2* mRNA, die vertaald worden in Cln1 en Cln2 eiwitten. Waarschijnlijk kunnen deze eiwitten samen met Cdc28 zorgen voor de activatie van Slt2, dat deel uitmaakt van de Pkc1-Slt2 celwand-integriteitsroute. Slt2 is betrokken bij veranderingen in de celwand die nodig zijn voor de vorming van de dochtercel. Cln1- en Cln2-Cdc28 complexen zorgen ook voor het aanzetten van DNA verdubbeling. De koppeling van DNA verdubbeling en celwandverandering zorgt ervoor dat een cel steeds zijn DNA verdubbeld als er ook een dochtercel gevormd wordt en andersom.

In hoofdstuk 3 staat beschreven hoe de lengte van de G1 fase verandert wanneer de hoeveelheid glucose of galactose (suikerflux) wordt verminderd. In normale, wild type cellen worden reservestoffen aangemaakt en de G1 fase verlengd wanneer de cellen gegroeid worden met een lage suikerflux. In gemuteerde cellen die geen reservestoffen kunnen maken, wordt de G1 fase ook niet verlengd. Tevens wordt in dit hoofdstuk beschreven hoe de niveaus van G1 cycline-eiwitten veranderen tijdens de G1 fase wanneer deze lang of kort is en of deze verandert wanneer cellen gevoed worden met galactose in plaats van met glucose. Als laatste wordt in hoofdstuk 3 beschreven dat Slt2 geactiveerd wordt aan het midden van de G1 fase en dat deze activatie doorloopt tot in de S fase tijdens groei met een hoge suikerflux. Wanneer cellen met een lage suikerflux gegroeid worden, bouwt de cel ook reservestoffen op. Deze veranderen de samenstelling in de cel al zodanig, dat de celwand aangepast moet worden en Slt2 wordt dan inderdaad in het midden van de G1 fase geactiveerd, maar deze activatie stopt niet in de S fase.

Het gebruik van gist in de levensmiddelenindustrie wordt steeds verder gemodificeerd om de opbrengst en smaak van de levensmiddelen te optimaliseren. Tijdens hun overleving en voortplanting hebben gisten voedingsbronnen zoals koolstof en stikstof nodig. Om gist verder te optimaliseren, moet eerst bekend zijn hoe de cel voedingsstoffen opneemt en verwerkt. In dit perspectief wordt ook gekeken naar de opname van glucose. De cel kan niet zomaar glucose opnemen, maar gebruikt hiervoor hexose transporter-eiwitten (Hxt's) die in de celmembraan zitten. Er zijn verschillende Hxt's in gist, maar er zijn er slechts 7 belangrijk, Hxt1,2,3,4,5,6 en 7. Deze belangrijke Hxt's zijn betrokken bij het opnemen van glucose. Dit glucose kan door de cellen meteen gebruikt worden om energie te krijgen, maar kan ook worden opgeslapen in de reservestoffen trehalose en glycogeen. Bij verschillende concentraties glucose in het medium komen verschillende Hxt's tot expressie. Alleen de expressie van *HXT5* wordt bepaald door de groeisnelheid van de cel, en niet door de hoeveelheid glucose in de omgeving.

In hoofdstuk 4 staat beschreven dat Hxt5 betrokken is bij de opbouw van de reservestof trehalose. Onder alle geteste omstandigheden werd *HXT5* tot expressie gebracht als trehalose werd opgebouwd. Belangrijk is vooral dat

HXT5 tot expressie wordt gebracht nadat de glucose in de omgeving was opgebruikt. Cellen maken dan zelf glucose vanuit bijvoorbeeld eiwitten of ethanol, via een proces dat gluconeogenese heet. In zoogdiercellen vindt de laatste stap van dit proces plaats in een organel dat het endoplasmatisch reticulum genoemd wordt. Een glucose transporter die verwant is aan Hxt5 transporteert daar de nieuwe glucose dan vanuit dit organel de 'vrije ruimte' van de cel, het cytoplasma, in. In gist, zit Hxt5 ook in het endoplasmatisch reticulum, wat erop wijst dat Hxt5 ook betrokken is bij de gluconeogenese. Hxt5 zit in een complex samen met het belangrijkste eiwit van de trehalose-opbouw, Tps1. Het bestaan van een complex dat de invoer van glucose (vanuit de omgeving de cel in) reguleert wordt voorgesteld en bestaat uit tenminste Hxt5 en Tps1. Dit complex is het 'glucose-response complex' genoemd, vrij vertaald het 'glucose-beantwoordend complex'.

In hoofdstuk 5 wordt beschreven welke functie het glucose-beantwooordend complex heeft in gehongerde cellen en hoe dit complex werkt. Wanneer Tps1 niet aanwezig is in cel, kan het glucose-beantwoordend complex niet functioneren. Wanneer gehongerde cellen dan nieuw glucose krijgen, wordt de invoer van glucose niet gereguleerd. Dit is wel nodig, omdat de eerste stappen waarbij glucose wordt omgezet in energie, eerst energie kosten in plaats van opleveren. De cellen die Tps1 missen, verbruiken al hun energie als ze glucose krijgen en gaan dood.

De expressie van de *HXT5* wordt anders gereguleerd dan de expressie van de andere belangrijkde Hxt's. Daarom is in hoofdstuk 6 beschreven via welke routes in de cel, Hxt5 wordt afgebroken. Hxt5 volgt dezelfde routes als de belangrijke Hxt's, maar de regulatie van de afbraak is waarschijnlijk anders. De belangrijke Hxt's hebben een bepaalde volgorde in de bouwstenen van het eiwit, dat de PEST-sequentie wordt genoemd. Het hebben van deze sequentie gaat vaak samen met een modificatie van het eiwit net voordat het wordt afgebroken. Dit heet ubiquitinering en daarbij worden één of meerdere ubiquitine (dit is ook een bepaald eiwit) moleculen aan het af te breken eiwit geplakt. Hoewel Hxt5 ook een PEST-sequentie heeft, wordt Hxt5 niet geubiquitineerd. Wel wordt Hxt5 op een andere manier gemodificeerd, namelijk met behulp van een fosfaat-groep. Deze modificatie is echter

waarschijnlijk niet betrokken bij de afbraak, omdat de meeste afbraak pas gebeurd als de fosfaat-groep alweer van Hxt5 af is. De aanwezigheid van de fosfaat-groep en het glucose-beantwoordend complex komen tegelijk voor, en daarom wordt gesuggereerd dat de fosfaat-groep misschien een regulerende rol heeft in het glucose-beantwoordend complex.

In hoofdstuk 7 zijn de resultaten zoals beschreven in dit proefschrift aan elkaar gekoppeld en in een breder perspectief geplaatst. De overeenkomsten van Hxt5 met een glucose transporter in zoogdiercellen toont aan, dat zelfs een mechanisme dat van belang is om een multi-cellulair organisme te allen tijden van voldoende glucose te voorzien, geconserveerd is vanaf het relatief eenvoudige organisme *Saccharomyces cerevisiae*.

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Curriculum Vitae

Denise van Suylekom werd geboren op 20 maart 1979 in Oosterhout. In 1997 behaalde zij haar diploma aan het St. Oelbert Gymnasium in Oosterhout In datzelfde jaar begon zij met haar studie Medische Biologie aan de Universiteit Utrecht. Tijdens haar studie werd een eerste stage gelopen bij Pedriatische Endocrinologie in het Wilhelmina Kinderziekenhuis in Utrecht onder begeleiding van Dr. Maarten Jansen en Drs. Harry Vermeer. Een tweede stage werd gelopen bij Celbiologie onder begeleiding van Dr. Peter van der Sluijs en Drs. Magda Deneka. In 2002 werd de studie voltooid en begon zij als assitent in opleiding aan de Universiteit Utrecht bij de vakgroep Cellular Architecture and Dynamics onder begeleiding van Prof. Dr. Johannes Boonstra en Dr. Bruno Humbel. Tijdens deze periode werd het promotieonderzoek zoals beschreven in dit proefschrift verricht. Vanaf half april 2006 is de schrijfster werkzaam bij YACHT als interim professional en is gedetacheerd bij DSM.