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Biochemical Characterization of Two Yeast Paralogous Proteins Mth1 and Std1

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Abstract of a Dissertation
Submitted to the Graduate Studies Office
of The University of Southern Mississippi
in Partial Fulfillment of the Requirements
for the Degree of Doctor of Philosophy

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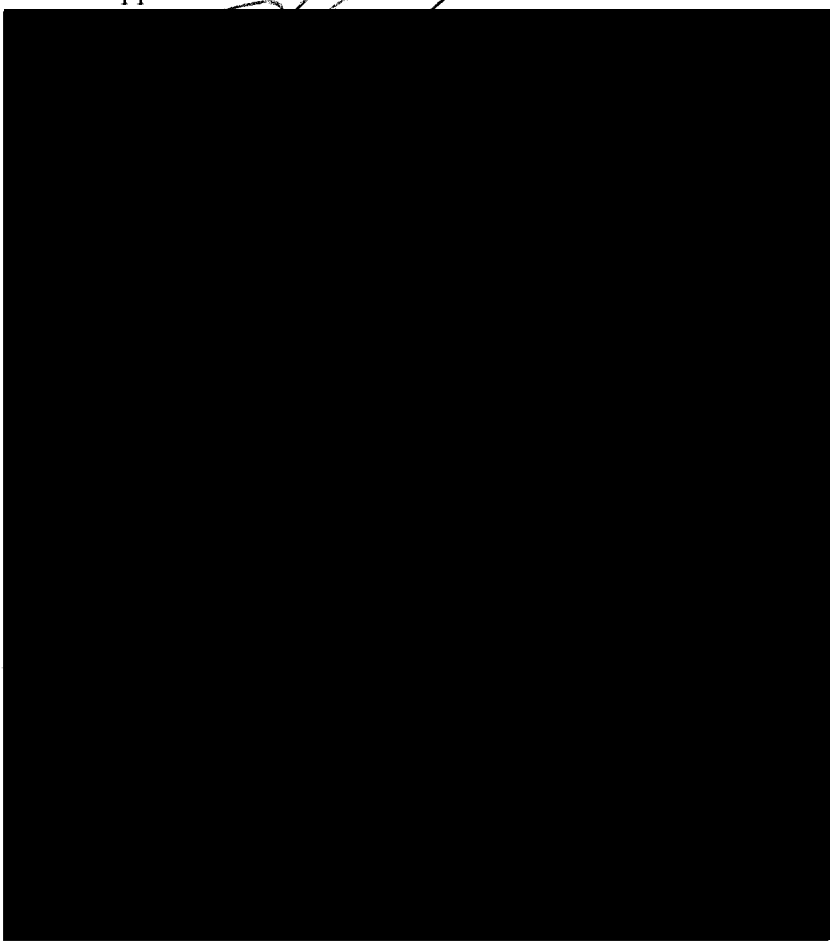

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ABSTRACT

BIOCHEMICAL CHARACTERIZATION OF TWO YEAST PARALOGOUS PROTEINS Mth1 AND Std1

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Glucose is the most abundant monosaccharide and preferred carbon and energy source for most cells. Many organisms have evolved sophisticated means to sense glucose and respond to it appropriately. The budding yeast, *Saccharomyces cerevisiae* senses glucose through two transmembrane proteins, Snf3 and Rgt2. In the presence of extracellular glucose Snf3 and Rgt2 generate an intracellular signal that leads to the degradation of Mth1 and Std1, thereby inducing the expression of hexose transporter genes *(HXT)* by inhibiting the function of Rgt1, a transcriptional repressor of *HXT* genes. Mth1 and Std1 are degraded via the Yck1/2 Kinase-SCF^{Grr1}-26S proteasome pathway triggered by the glucose sensors. *RGT2-1* and *SNF3-1* induce expression of *HXT* genes even in the absence of glucose. I show that *RGT2-1* promotes ubiquitination and subsequent degradation of Mth1 and Std1 regardless of the presence of glucose. Site-specific mutagenesis reveals that conserved lysine residues of Mth1 and Std1 might serve as attachment sites for ubiquitin, and that the potential casein kinase (Yck1/2) consensus sites in Mth1 and Std1 are needed for their phosphorylation. The data provides biochemical evidence for glucose independent degradation of Mth1 and Std1. I further identified, the subcellular localization and the cellular compartment in which of Mth1 and Std1 are degraded in response to glucose. The data shows that, Mth1 and Std1 are present

in nucleus when they are not degraded due to mutational blocks in the Snf3/Rgt2-Rgt1 pathway. Mth1 and Std1 could be degraded in both the nucleus and cytoplasm when its subcellular localization is artificially manipulated; however, glucose-induced degradation occurs only in the nucleus. I also demonstrate that membrane tethering of Yck1/2 plays no or little role in the degradation of Mth1. Transcriptomic analysis of *mth1Δstd1Δ* mutant identified new target genes for Mth1 and Std1 in new functional categories including mitochondrial/respiration genes, transporter genes and amino acid pathway genes in addition to *HXT* genes. This analysis provided insights into understanding the new functions of the two paralogous proteins Mth1 and Std1.

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CHAPTER 1

BACKGROUND AND SIGNIFICANCE

The budding yeast *Saccharomyces cerevisiae*, a unicellular eukaryote is an excellent model organism for studying basic biological processes. Being a eukaryote, *S. cerevisiae* has a nucleus containing chromosomes and the cells divide in a similar manner to human cells and also share many other biological processes. Yeast proteins share homology with the proteins of higher eukaryotic organisms including *Drosophila melanogaster*, *Caenorhabditis elegans* and *Homo sapiens.* Budding yeast has a rapid growth and is also easy to grow. The average cell cycle of budding yeast is 90 minutes, much shorter when compared to human cells. Glucose is the preferred carbon source for yeast and many other micro-organisms. Furthermore, glucose is also the prime carbon and energy source in higher multicellular organisms. Therefore, glucose-sensing and signaling in these organisms is of vital importance for maintenance of sugar homeostasis (Rolland *et al.* 2001). Genetic manipulations can be easily made in yeast and as large variety of examples provide evidence that substantial cellular functions are highly conserved from yeast to mammals, I have used *S. cerevisiae* to study glucose sensing and signaling, specifically Rgt2/Snf3-Rgt1 glucose induction pathway. In this chapter, I review the current literature relevant to the understanding of this work.

Glucose Sensing and Signaling in *S. cerevisiae*

In free living microorganisms there is constant change of environment around them and nutrient availability is the major factor controlling growth and development. The successful survival of organisms requires adaptation to the changes in environment.

Cells respond to the changes in environment by a variety of signaling pathways, among them two are prominent. In the first pathway, a protein in the plasma membrane binds to a nutrient or hormone that activates a cascade of reactions affecting metabolic or regulatory enzymes, transcription factors, etc. (Forsberg and Ljungdahl 2001; Kroeze *et al.* 2003). The second pathway depends on the uptake of the nutrient and on its metabolism, which causes change in the concentration of intracellular metabolites with a regulatory function (Gancedo 2008). The metabolites may in turn interact with different kinds of proteins and modify their regulatory functions, which may include their enzymatic activity or binding to different proteins or binding to specific regions and modulating the transcription rate of the corresponding genes (Sellick and Reece 2005). Glucose is the preferred carbon and energy source for *S. cerevisiae.* The presence of glucose triggers a wide variety of regulatory processes which are required for the optimal utilization of the preferred carbon source. The major physiological changes caused by glucose include, 1. Change in the concentration of intracellular metabolites (Kresnowati *et al.* 2006); 2. Modifications and eventual degradation of some enzymes (Serrano 1983); 3. Alterations in the stability of a number of mRNAs (Mercado *et al.* 1994) and 4. Transcription of different genes is either repressed or induced (Gancedo 1998; Johnston 1999; Wang *et al.* 2004). Because of the above changes in metabolism there is an increase in growth rate of the yeast (Johnston *et al.* 1979). The major glucose signaling pathways will be discussed in the next section.

Glucose Sensing and Signaling Pathways in *S. cerevisiae*

Snf1-Mediated Glucose Repression Pathway

Glucose repression is mainly responsible for the down regulation of respiration, gluconeogenesis, the transport and catabolic capacity of non-fermentable sugars during the growth on glucose. The Snf1 protein kinase (also called as Cat1, Ccr1) is the central component of the glucose repression signaling pathway and is required for transcription in response to glucose repression. *SNF1* was first identified in screens for regulatory factors in the glucose response (Ciriacy 1977; Entian and Zimmermann 1982). The Snf1 kinase regulates repression by Mig1, a DNA-binding repressor protein, which recruits the Ssn6 (Cyc8)-Tup1 co-repressors to the promoter of many glucose repressed genes (Treital and Carlson 1995; and Figure.1). A major function of the Snf1-kinase is to inhibit repressor function of Mig1 when glucose is limiting. Snf1 is involved in the localization of Mig1 repressor and thus controlling its repressor functions. In high glucose conditions Mig1 is in the nucleus (Figure. 1) and represses genes required for the metabolism of alterative carbon sources (non-fermentable carbon sources). In low glucose conditions Snf1 is active and phosphorylates Mig1 which then leaves the nucleus (Figure. 1) and the genes like *SUC2*, *MAL*, *CAT8* etc, are derepressed (De vit *et al.* 1997; Treitel *et al.* 1998).

The Reg1/Glc7 acts as negative regulator of Snf1 kinase. In high glucose, the Snf1 kinase complex is inactive and Snf1 regulatory domain autoinhibits the catalytic domain. In low or no glucose, this autoinhibition is relieved and the Snf4 activating subunit binds the regulatory subunit. The Glc7-Reg1 protein phosphatase associates with the activated complex and appears to facilitate the transition back to the inactive state

(Ludin *et al.* 1998). In *reg1* mutant the Snf1 kinase becomes trapped in the activated state.

Glucose Repression and the Role of Hxk2

Hxk2 along with Hxk1 and Glk1 are involved in the intracellular phosphorylation of glucose converting it into glucose-6-phosphate. Of these three enzymes only Hxk2 is highly expressed in the presence of glucose (Herrero *et al.* 1995). *HXK2* mutations show defect in glucose repression (Entian 1980; Entian and Mecke 1982). The exact role of Hxk2 in glucose signaling pathway has been debated for long. It has been now agreed that the catalytic activity of Hxk2 does not play a significant role in the intracellular glucose sensing pathway (Moreno and Herrero 2002). One model postulates that after the onset of phosphoryl transfer reaction, a stable transition intermediate alters Hxk2 conformation and mediates its regulatory function in altering target gene expression (Bisson and Kunathigan 2003; Kraakman *et al.* 1999). Approximately 14 percent of Hxk2 was observed in the nucleus in glucose grown cells. The discovery suggested a regulatory involvement of this hexokinase in response to glucose. Even though Hxk2 was found to be in nucleus in the presence of glucose, there were contradictory results regarding the region of Hxk2, necessary for its nuclear localization in the glucose grown cells (Herrero *et al.* 1998; Rodriguez *et al.* 2001). Contrary results were also reported for phosphorylatable Ser 14 residue in Hxk2, it was reported either to mediate or not to mediate glucose repression (Herrero *et al.* 1998; Mayordomo and Sanz 2001). More evidence is needed to show the definite role of Hxk2 in glucose signalling pathway.

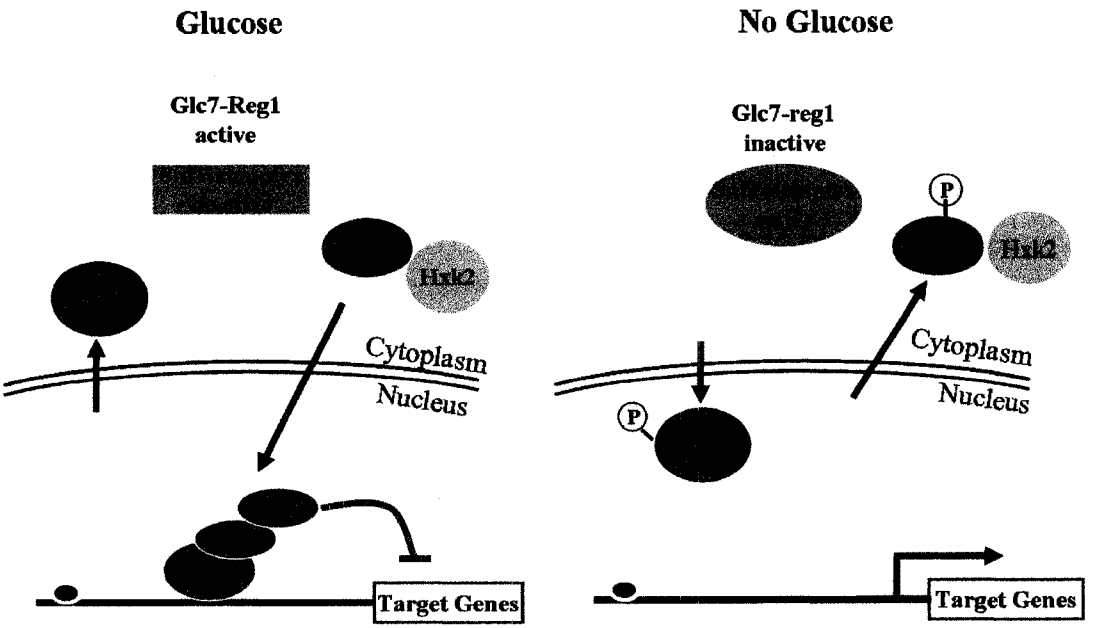


Figure.1 Glucose repression in *S. cerevisiae*. In presence of glucose the repressing protein Mig1 is mainly in its unphosphorylated form due to protein phosphatase activity of the Glc7-Reg1 complex, coupled with a low activity of the Snf1 complex. Unphosphorylated Mig1 is nuclear and cooperates with Hxk2 to repress transcription of target genes (~1000 genes including *SUC2*, *MAL* and *CAT8*) in co-ordination with general transcription factors Ssn6 and Tup1. When glucose is depleted, the Snf1 kinase is phosphorylated by upstream kinases. Upon phosphorylation the Snf1 kinase complex becomes active and localizes to the nucleus, where it phosphorylates Mig1. Phosphorylated Mig1 becomes inactive and leaves the nucleus along with Hxk2 and derepresses the target genes.

Glucose Sensing via Ras/cAMP/PKA Pathway

cAMP, an intracellular metabolite plays an important role in glucose signalling in yeast (Gancedo 2008). Glucose causes activation of the GTP-binding proteins Ras2 and Gpa2, leading to an increase in the cAMP levels (Toda *et al.* 1985; Nakafuku *et al.* 1988), which stimulates the cAMP-dependent protein kinases Tpk1, Tpk2 and Tpk3 (Toda *et al.* 1987). The GTP bound G-proteins (Ras and Gpa2) bind independently to adenylate cyclase (Cyr1) and stimulate the production of cAMP (Santangelo 2006). Heterotrimeric G-proteins are signalling molecules composed of α, β and γ subunits. Gpa2, a member of

α family of heterotrimeric G proteins regulates cAMP levels. It is associated with Gpr1 which is member of G protein-couple receptor super family and it has seven transmembrane domains (Santangelo 2006).

The small GTP-binding proteins, Ras1 and Ras2, play a role in the cell's adaptation to glucose by coupling cyclic AMP (cAMP) production to the presence of glucose in the medium (Wang *et al.* 2004). Ras G proteins are attached to the plasma membrane via a palmitoyl moiety. Ras is a part of Ras/Cyr1 complex. Other components of this complex are RasGEFs (Cdc25 and Sdc25) and RasGAPs (Ira1 and Ira2). They regulate adenylate cyclase by controlling Ras switch. Ras proteins are inactive in their GDP-bound form and active in GTP-bound form. The production of cAMP activates PKA. PKA has regulatory subunits (Bcy1) and catalytic subunits (Tpk). When bound to the kinase subunits (Tpk) regulatory Bcy1 subunits keep PKA inactive. PKA is activated by binding of cAMP to the Bcy1 subunits and releasing Tpk subunits from the complex. Active PKA can phosphorylate a number of proteins involved in transcription, energy metabolism, cell cycle progression and accumulation of glycogen and trehalose.

Snf3/Rgt2 Mediated Glucose Induction Pathway

This pathway of glucose regulation of gene expression helps ensure that yeast can live well on glucose. Yeast growing on high levels of glucose obtains most of their energy by fermentation (Lagunas 1979) which produces only a few ATPs for one glucose molecule used. For this reason they need to pump large amount of glucose through glycolysis to generate enough energy for efficient survival. Glucose induces the expression of most genes encoding for glycolytic enzymes and *HXT* genes encoding

hexose transporters. Glucose induction of *HXT* genes by Rgt2/Snf3-Rgt1 pathway is the focus of my dissertation and I will discuss more about the components of glucose induction pathway later in this chapter.

Glucose induction of *HXT* expression is triggered by plasma membrane sensor proteins Snf3 and Rgt2 (Ozcan *et al.* 1996 and Figure. 2). In presence of extracellular glucose Snf3 and Rgt2 trigger a signal transduction pathway, which first activates type I casein kinases Yck1 and Yck2 (Moriya and Johnston 2004). The activated kinases phosphorylate regulatory proteins Mth1 and Std1 (Moriya and Johnston 2004). Mth1 and Std1 interact with Rgt1 (Tomas-Cobos and Sanz 2002; Lakshmanan *et al.* 2003), a transcriptional repressor of genes induced by glucose (Flick *et al.* 2003; Kim *et al.* 2003; Palomino *et al.* 2005). Phosphorylated Mth1 and Std1 are subject to ubiquitination by SCF^{Grr1}-ubiquitin ligase which targets them for degradation by 26S proteasome (Spielewoy *et al.* 2004; Kim *et al.* 2006). Degradation of Mth1 and Std1 relieves Rgt1 from the promoters of hexose transporter genes causing an induction of their expression which mediates the transport of glucose into the cell.

Induction of *HXT* expression occurs independent of glucose in dominant mutants *RGT2-1* and *SNF3-1* (Ozcan *et al.* 1996; Ozcan *et al.* 1998). However, it is not shown if the induction of *HXT* expression occurs by the degradation of the paralogous proteins Mth1 and Std1. The goal of my first project is to provide biochemical evidence for the glucose-independent induction *HXT* genes expression by looking at degradation of Mth1 and Std1 in *RGT2-1* and *SNF3-1* (Chapter IV). Mth1 and Std1 in the presence of glucose are phosphorylated by casein kinases Yck1 and Yck2 which are attached to plasma membrane. This requires Mth1 and Std1 to move out of nucleus for them to be

phosphorylated and ultimately degraded. In my second project (Chapter V), I have looked at the localization of Mth1 and Std1 and the subcellular compartment in which they are degraded in response to glucose. I show here that Mth1 is not excluded from the nucleus in response to glucose. The protein was found in the nucleus when it is not degraded due to mutational blocks in the pathway. We also found that Mth1 could be degraded in both nucleus and cytoplasm when its subcellular localization is artificially manipulated; however, glucose induced degradation occurs only in the nucleus. I also provide evidence supporting that membrane tethering of Yck1/2 is not essential for the glucose induced degradation of Mth1 and Std1.

Mth1 and Std1 serve as transcriptional regulators of *HXT* genes in conjunction with the repressor protein Rgt1. In order to find out other cellular functions of Mth1 and Std1, I did transcriptomic analysis (Chapter VI) using *mth1Δstd1Δ* mutant and identified different functional categories (in addition to hexose transporter genes) of target genes regulated by Mth1 and Std1. The identified target genes included genes with and without Rgt1 binding sites in their regulatory regions (Chapter VI). In the later half of this chapter I review the pertinent literature about the components involved in glucose induction pathway mediated by Snf3/Rgt2 glucose sensors.

The Snf3 and Rgt2 Glucose Sensors

Snf3 and Rgt2 are plasma membrane proteins with 12 transmembrane domains and they belong to hexose transport family. *SNF3* gene was identified in a screen for mutants deficient in the utilization of raffinose, based on the inability to derepress the invertase encoding *SUC2* gene (Niegeborn and Carlson 1984; Neigeborn *et al.* 1986).

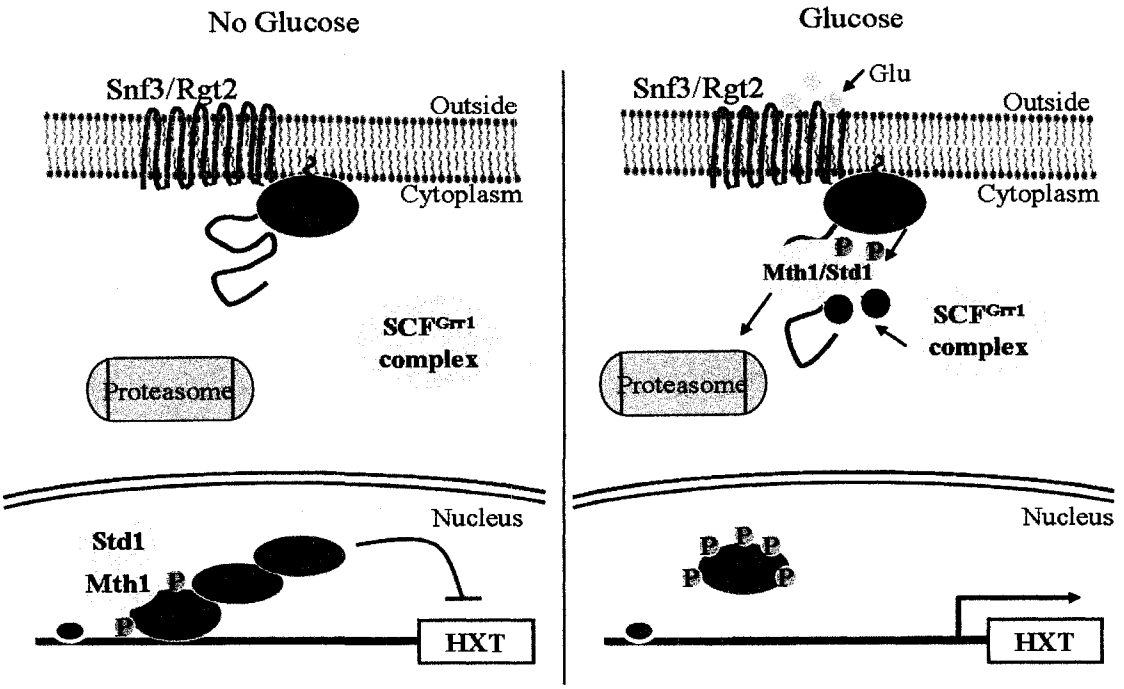


Figure 2. Snf3/Rgt2 mediated glucose signaling pathway. In the absence of glucose, the repressing complex including Rgt1, Mth1/Std1, Ssn6 and Tup1 binds to the promoters of the *HXT* genes and blocks their transcription. When glucose is present, it binds to the Snf3/Rgt2 sensors, thus activating the membrane bound casein kinase 1 (Yck1/2). Activated Yck1/2 phosphorylates Mth1/Std1, bound to the C-terminal tails of Snf3 and Rgt2. Phosphorylated Mth1/Std1 are recognized by the SCF^{Grr1} complex, which tags them through ubiquitination, to be degraded by the proteasome. Removal of Mth1/Std1 allows the phosphorylation of Rgt1, which dissociates from the promoter, allowing derepression of *HXT* genes.

The *RGT2* was isolated as dominant mutant clone *RGT2-1,* that bypasses the requirement of Snf3 for the growth on low concentrations of glucose by restoring high-affinity transport. Snf3 and Rgt2 are 60% similar to each other and about 30% similar to other glucose transporters (Ozcan *et al.* 1996). They differ from hexose transporters in possessing long C-terminal tails and also they lack the ability to transport glucose. The long C-terminal tails are present in the cytoplasmic side of the plasma membrane and they play an important role in glucose signaling, (Ozcan *et al.* 1998; Dlugai *et al.* 2001) but are not an absolute requirement (Moriya and Johnston 2004). Snf3 serves as a sensor

for low levels of glucose (high affinity glucose sensor), as it is needed for the induction of *HXT* genes by low glucose. Rgt2 serves as the sensor for high levels of glucose (low affinity glucose sensor), because it is required for the maximal induction of *HXT1* by high glucose (Ozcan *et al.* 1996).

RGT2-1 and SNF3-1 Promote Glucose Independent Induction of HXT Genes

Snf3 and Rgt2 serve as receptors of extracellular glucose and generate a signal inside the cell that is required for the induction of *HXT* gene expression. Interestingly, dominant mutations in glucose sensors, *SNF3-1* and *RGT2-1* generate a constitutive glucose signal leading to the expression of *HXT* genes even in the absence of glucose. This suggests that the mutated proteins are locked in a conformation similar to glucose bound forms of Snf3 and Rgt2, independent of carbon source present in the medium. The dominant mutations in Rgt2 (*RGT2-1*) or Snf3 (*SNF3-1*) change an arginine residue at 231 or 229, respectively to a lysine residue (Ozcan *et al.* 1996). It is not known how *RGT2-1* and *SNF3-1* cause induction of *HXT* genes expression in the absence of glucose. It is assumed that they do so by causing the degradation of Mth1 and Std1 proteins as it occurs in case of glucose signalling. Verification of glucose-independent degradation of Mth1 and Std1 is essential to avoid defining incorrect models. For instance, we cannot rule out the possibilities that: 1) *RGT2-1* and *SNF3-1* simply inactivate Rgt1 (there is ample evidence that Mth1 and Std1 work together with Rgt1, the *HXT* repressor); 2) *RGT2-1* and *SNF3-1* promote nuclear export of Mth1 and Std1. In these two models, expression of *HXT* genes is still induced without the degradation of Mth1 and Std1.

Hence, in chapter IV I provide biochemical evidence showing that *RGT2-1* and *SNF3-1* induce degradation of Mth1 and Std1 even in the absence of glucose.

Role of Yeast Casein Kinases Yck1 and Yck2 in Glucose Signaling

Snf3 and Rgt2 in presence of glucose generate a signal which is thought to activate Yck1 and Yck2, which in turn phosphorylate Mth1 and Std1 (Moriya and Johnston 2004). Casein kinase 1 is the name given to Ser/Thr protein kinase activity found in eukaryotic cells (Robinson *et al.* 1993). Yeast casein kinases Yck1 and Yck2 are functional homologues and loss of *YCK* function is lethal (Robinson *et al.* 1992). *YCK1* was isolated as a suppressor of the requirement for *SNF4* function, and *YCK2* was isolated by its ability to act as a suppressor of high salinity intolerance (Robinson *et al.* 1992). Yck1 and Yck2 are tethered to the membrane via palmitate moieties attached to the C-terminal Cys-Cys sequences and Akr1, a palmitoyl transferase protein is required for the membrane tethering of Yck1 and Yck2 (Feng and Davis 2000). Casein kinase 1 phosphorylates the serine residue at the consensus target sequence SXXS. Mth1 and Std1 are phosphorylated at the serine residues in SXXS consensus sites by Yck1 and Yck2 which are then degraded by via Grr1-dependent mechanism.

Membrane Tethering of Yck1 and Yck2 Requires Akr1

Many signaling proteins tether to membrane sites through lipid modifications, i.e. palmitoylation, myristoylation or prenylation (Roth *et al.* 2002). Palmitoylation involves the thioesterification of cysteine by palmitic acid and often directs the modified protein to the plasma membrane (Roth *et al.* 2002). Akr1 is polypotic membrane protein containing

DHHC cysteine-rich domain (CRD) and is a palmitoyl transferase (PTase). *AKR1* encodes an 86-kd protein with six predicted trans-membrane domains, six ankyrin repeat sequences mapping to the amino-terminal hydrophilic domain, and a DHHC-CRD sequence mapping between trans-membrane domain four and five (Roth *et al.* 2002). Akr1 is required for the proper localization of the type I casein kinase Yck2 (Feng and Davis 2000). The membrane association of Yck1 and Yck2 depends apparently on the lipid modification of COOH-terminal Cys-Cys sequences (Vancura *et al.* 1994). Yck2 is mislocalized to cytoplasm in *akr1Δ* cells as seen with the cis-mutation of the Yck2 C-terminal cysteines (Feng and Davis 2000).

Role of SCF^{Grr1} Complex in Glucose Signaling

In the presence of glucose Mth1 and Std1 are phosphorylated by Yck1 and Yck2 which are then subject to ubiquitination by *SCF^{Grr1} complex* and degraded by 26S proteasome (Flick *et al.* 2003; Kim *et al.* 2006). Degradation of proteins is triggered by the covalent attachment of ubiquitin onto lysine residues of substrates, which targets them for destruction by 26S proteasome (Ciechanover and Schwartz 2002). The enzyme classes required for these reactions include; 1) E1 ubiquitin-activating enzyme, 2) the E2 ubiquitin conjugating enzymes and 3) E3 ubiquitin ligases (Peters 1998). The anaphase-promoting complex (APC) and Skp1-Cullin-F-box protein complex (SCF) are multiprotein E3 ligases. SCF family of enzymes form stable complex with an E2 enzyme, most commonly Cdc34, and contains several common components: a scaffold protein (also called cullin), a RING-finger protein Hrt1 and an adaptor protein Skp1 (Flick *et al.* 2003; Patton *et al.* 1998; Seol *et al.* 1999). In addition they contain a variable F-box

protein which confers substrate specificity to the SCF complex (Bai *et al.* 1996; Skowyra *et al.* 1997). Typically, F-box proteins have a bipartite structure consisting of two regions, 1) F-box domain interacting with SCF via Skp1 and 2) a substrate recognition motif, such as a leucine-rich repeat (LRR) domain or WD40 repeat domain which participates in substrate binding (Bai *et al.* 1996; Skowyra *et al.* 1997). In SCF^{Grr1} complex Grr1 serves as F-box protein which contains a large substrate binding domain built on 12 LRRs (Kishi *et al.* 1998; Li and Johnston 1997). SCF^{Grr1} mediates the degradation of G1 cyclins Cln1 and Cln2 (Barral *et al.* 1995; Seol *et al.* 1999; Skowyra *et al.* 1997), bud emergence protein Gic2 (Jaquenoud *et al.* 1998), *HXT*'s co-repressors Mth1 and Std1 (Flick *et al.* 2003) and cytokinesis septum forming protein Hof1 (Blondel *et al.* 2005). Deletion of *GRR1* (glucose repression resistant) abolishes the induction of *HXT* gene expression even in the presence of glucose. Grr1 is required for the hyper-phosphorylation of Rgt1 and its dissociation of Rgt1 from the promoters of *HXT* genes. However Rgt1 is not a direct target of ubiquitination by SCF^{Grr1} complex. Grr1 inactivates Mth1 and Std1 in response to glucose and their inactivation occurs at the level of degradation (Flick *et al.* 2003). Glucose-independent degradation of Mth1 and Std1 by *RGT2-1* and *SNF3-1* requires SCF^{Grr1} and also lysine residues in these proteins (Chapter IV).

Hexose Transporters in *S. cerevisiae*

Glucose in addition to being a major nutrient acts as a "growth hormone" to regulate several aspects of cell growth, metabolism and development (Ozcan and Johnston 1999). The first and limiting step of glucose metabolism is its ability to

transport across the plasma membrane (Ozcan and Johnston 1999). There are 20 genes in *Saccharomyces cerevisiae* that encode proteins similar to hexose transporters, which are *HXT1* to *HXT17*, *GAL2*, *SNF3*, and *RGT2* (Bisson *et al.* 1993; Ciriacy and Reifenberger 1997; Kruckeberg 1996). The hxt proteins belong to the major facilitator superfamily (MFS), which transport their substrates by passive, energy-independent facilitated diffusion with glucose moving down a concentration gradient (Bisson *et al.* 1993). Prokaryotes and mammals have many sugar transporters that belong to MFS family of transporter. Studies performed with yeast hexose transporters will be valuable in understanding the structure, function and regulation of glucose transporters from a wide variety of other organisms (Ozcan and Johnston 1999).

Sugar transporters in *Saccharomyces cerevisiae* are of two categories: low affinity transporters and high affinity transporters. The presence of different affinity glucose transporters helps yeast to grow on a broad range of glucose concentrations (from few μM to 2M). Based on the amount of glucose available, appropriate glucose transporters are expressed which is regulated by *HXT* gene expression (Ozcan and Johnston 1999). Of the 20 members of the *HXT* gene family only seven are known to encode functional glucose transporters. *hxt* null mutant strain which lacks seven *HXT* genes (*hxt1Δ-hxt7Δ*) fails to grow on glucose, fructose, or mannose and has no glycolytic flux (Boles and Hollenberg 1997; Liang and Gaber 1996; Reifenberger *et al.* 1997). Expression of any one of the seven *HXT* genes into *hxt* null strain is sufficient to allow growth on glucose. *HXT2* and *HXT4* encode high affinity glucose transporters, which are sufficient for growth on 0.1% of glucose. *HXT1* enables growth only on high glucose concentrations (more than 1%). Therefore *HXT1* encodes low affinity glucose transporters (Reifenberg

et al. 1997). Gal2, a galactose transporter is also able to complement the glucose growth defect of *hxt* null mutant (Liang and Gaber 1996). *HXT8* through *HXT17* encode proteins that either are unable to transport glucose or not expressed under the conditions tested (Ozcan and Johnston 1999). *S. cerevisiae* cells developed mechanisms to express only the glucose transporters appropriate for the amount of extracellular glucose available. This is achieved by the combined regulatory mechanisms, including transcriptional regulation of various *HXT* genes in response to extracellular glucose (Ozcan and Johnston 1995; Ozcan *et al.* 1996a; Wendell and Bisson 1994) and inactivation of Hxt proteins under certain conditions (Boles and Hollenberg 1997; Horak and Wolf 1997; Krampe *et al.* 1998).

Transcriptional Regulation of HXT Gene Expression by Glucose

Transcription of the main four *HXT* genes (*HXT1-HXT4*) is induced 10 to 300 fold depending on the gene (Ozcan and Johnston 1995). The different regulation mechanisms to control the expression of these four genes include; 1) induction by glucose, independent of sugar concentration (*HXT3*), 2) induction by only low levels of glucose (*HXT2* and *HXT4*), and 3) induction only by high concentrations of glucose (*HXT1*). These three responses to glucose are due to the action of three overlapping regulatory pathways (Figure. 3, adapted from Ozcan and Johnston 1999).

HXT6 and *HXT7* genes are regulated similarly and they encode nearly identical proteins. *HXT6* expression is repressed by high concentration of glucose (Liang and Garber 1996). *HXT6* expression is high in cells growing on non-fermentable carbon sources such as ethanol and glycerol and induced only two to three folds in presence of

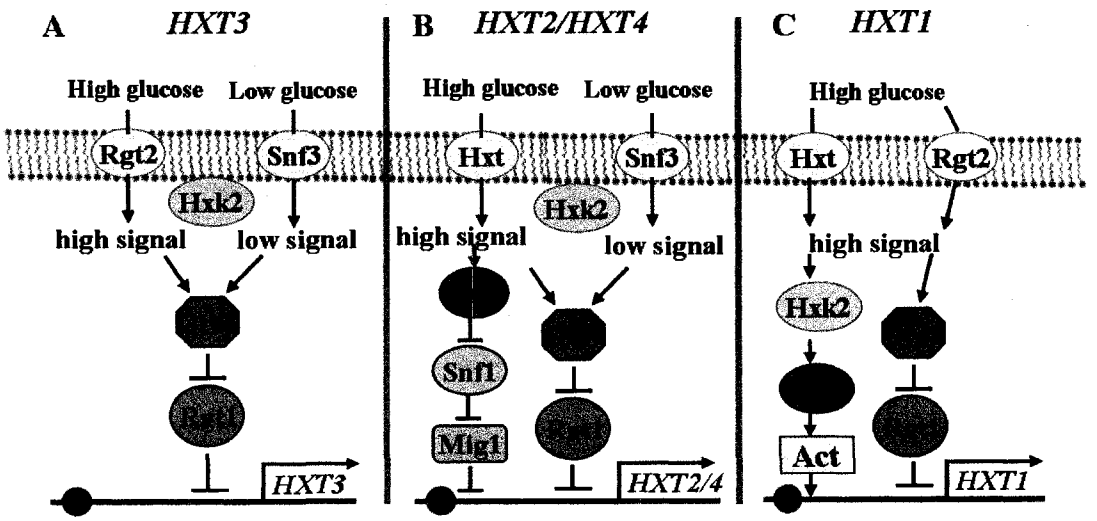


Figure 3. Three different modes of induction of *HXT* gene transcription by different levels of glucose. Figure adapted from Ozcan and Johnston, 1999. An arrow implies positive regulation; a line with bar denotes negative regulation. Glucose induction of all four genes is due to the repression mechanism by Rgt1, which inhibits expression of *HXT* genes in the absence of glucose. (A) Both high and low concentrations of glucose induce *HXT3* by inhibiting Rgt1 repressor function. The intracellular glucose signal responsible for Grr1-mediated inhibition repression function is generated by Snf3 and Rgt2, which serve as glucose sensors for low and high concentrations of glucose, respectively. (B) *HXT2* and *HXT4* are subject to glucose repression by Mig1 repressor, and the Snf1 protein kinase, which regulates Mig1 function. Superimposition of this regulatory pathway at the *HXT2* and *HXT4* promoters results in the induction of these genes by low level concentrations of glucose. (C) *HXT1* is only induced by high concentrations of glucose.

low levels of glucose or raffinose. Only limited information is available on the expression of remaining *HXT* genes (*HXT5* and *HXT8* to *HXT17*). These *HXT* genes excluding *HXT5* and *HXT13* are expressed at very low levels, being expressed 30 to 300 fold less than *HXT1* and *HXT2*. All these genes are subjected to several different modes of regulation by glucose (Ozcan and Johnston 1999). Regulation of *HXT5* and *HXT13* expression is similar to that of *HXT2* and *HXT4*, except *HXT5* repression is not as strong in high glucose and *HXT13* induction is only about four fold in low glucose (Ozcan and Johnston 1999).

Transcriptional Regulation of SNF3 and RGT2 Genes

Snf3 and Rgt2 are about 30% similar to the other members of the Hxt family (Kruckeberg 1996) and do not transport glucose but serve as sensors of extracellular glucose that generate the intracellular signal for the induction of *HXT1* to *HXT4* expression. *SNF3* and *RGT2* are expressed 100 to 300 fold lower than *HXT1* to *HXT4* genes (Ozcan *et al.* 1996). Snf3 is a high affinity glucose sensor and hence the transcription of *SNF3* is repressed at high concentration of glucose (Marshall-Carlson *et al.* 1990; Neigeborn *et al.* 1986; Ozcan and Johnston 1995). Rgt2 functions as low affinity glucose sensor and the expression of *RGT2* is independent of glucose concentration (Ozcan *et al.* 1996).

Rgt1: A Transcriptional Factor in Glucose Induction Pathway

RGT1 gene was isolated as a gene whose inactivation suppresses the high affinity glucose transport defect of *snf3* mutants (Erickson and Johnston 1994; Marshall-Carlson *et al.* 1991 and Vallier *et al.* 1994). Rgt1 binds to *HXT* gene promoters and inhibits their expression in the absence of glucose (Mosley *et al.* 2003; Flick *et al.* 2003 and Kim *et al.* 2003). However, with the appearance of glucose, Rgt1 becomes hyperphosphorylated (Mosley *et al.* 2003; Flick *et al.* 2003; Kim *et al.* 2003) and is unable to bind to *HXT* gene promoters (Kim *et al.* 2003), which leads to the induction of *HXT* gene expression. Rgt1 belongs to the Zn_2Cys_6 family of transcription factors and contains an amino-terminal zinc cluster as the DNA binding domain (Ozcan *et al.* 1996a). Gal4 and other members of this transcription factors family bind as dimer to two CGG repeats separated by specific number of nucleotides and the number of nucleotides is different for each transcription

factor (Marmorstein *et al.* 1992; Ozcan *et al.* 1996a). However Rgt1 binds as a monomer and its binding site contains only one CGG repeat (Ozcan and Johnston 1996; Ozcan *et al.* 1996a). Unlike Gal4 and other transcription factors, Rgt1 lacks the coiled coil dimer required for dimerisation. *HXT* gene promoters contain multiple binding sites for Rgt1 with the consensus sequence 5'-CGGANNA-3' which act synergistically in repression of *HXT* gene transcription (Kim *et al.* 2003) and also the prescence of altleast five copies of Rgt1 binding site is required for repression by Rgt1 (Kim *et al.* 2003).

Rgt1 functions as a repressor in the absence of glucose. In addition, Rgt1 functions as an activator of transcription at high concentrations of glucose (Ozcan *et al.* 1996a). In prescence of high glucose Rgt1 gets hyperphosphorylated and loses its ability to bind to *HXT* gene promoters *in vitro* and *in vivo* (Kim *et al.* 2003). Rgt1 has also been shown to bind to the promoters of *SUC2-B* promoter region (Hazbun and Fields 2002). Deletion of *RGT1* reduces the induction of *SUC2* gene expression by low levels of glucose, suggesting that Rgt1 may function as activator of *SUC2* expression (Hazbun and Fields 2002; Ozcan *et al.* 1997).

General Transcription Repressors, Ssn6 and Tup1

Ssn6-Tup1 complex represses the expression of several different genes (Gancedo 1998; Smith and Johnston 2000). This complex is also required for the repression of *HXT* gene expression by Rgt1 when glucose is absent (Ozcan and Johnston 1995; Ozcan *et al.* 1996b). There is recent data which suggests Ssn6 interacts with Rgt1 (Tomas-Cobos and Sanz 2002) and that Ssn6 associates with the promoters of *HXT* genes (Kim *et al.* 2003). In *rgt1Δ* mutant, Ssn6 is unable to associate with the *HXT* gene promoters suggesting

that the Ssn6 association is dependent on Rgt1. Like Ssn6-Tup1, Mth1 and Std1 are also required for the transcription repression of *HXT*, it is possible that they exist in a complex. The other possibility is that Mth1 and Std1 may be required to target the Ssn6-Tup1 complex to the *HXT* gene promoter to establish their repression in the absence of glucose (Mosley *et al.* 2003). Tup1 has been proposed to repress gene expression by two different mechanisms; 1) Tup1 interacts with mediator complex (Gromoller and Lehming 2000; Papamichos-Chronakis *et al.* 2000; Zaman *et al.* 2001) and 2) by deacetylation of histones at specific promoters (Watson *et al.* 2000; Wu *et al.* 2001). The exact role of Tup1 and Ssn6 in repression of the *HXT* gene expression by Rgt1 is not known.

Mth1 and Std1 are the Co-repressors of HXT Gene Expression

Mth1 and Std1 are paralogous proteins and they are 61% identical (Hubbard *et al.* 1994 and Figure. 4) which interact with Rgt1 and repress *HXT* genes induced by glucose (Kim *et al.* 2003; Flick *et al.* 2003; Palomino *et al.* 2005). This interaction prevents dissociation of a repression complex formed by Rgt1, Mth1/Std1 and Ssn6 and Tup1 from the promoters *HXT* genes in the absence of glucose (Polish *et al.* 2005). *STD1* (also called as *MSN3*) was originally isolated as a muliticopy suppressor of the *snf* (sucrose non-fermenting) phenotype of a *snf4* mutant by a partial relief of *SUC2* repression (Hubbard *et al.* 1994; Tillman *et al.* 1995). *MTH1*, homolog of *STD1* is allelic to the genes *HTR1*, *DGT1* and *BPC1* for which dominant mutant alleles have been isolated previously (Gamo *et al.* 1994; Schulte *et al.* 2000). A dominant mutation in *MTH1* isolated as *HTR1-23* or as *DGT1-1* causes constitutive repression of the *HXT* genes independent of the presence of glucose (Ozcan *et al.* 1994; Gamo *et al.* 1994; Schulte *et*

al. 2000). The mutation in Htr1-23 changes isoleucine of Mth1 to either an aspartate or serine (Schulte *et al.* 2000). This mutant form of Mth1 is stable, independent of carbon source and thus causes constitutive repression of *HXT* genes. Std1 interacts with Rgt1 *in vivo* (Tomas-Cobos and Sanz 2002) and the interaction of Rgt1 with Std1 or Mth1 occurs only in the absence of glucose (Lakshmanan *et al.* 2003). Agreeing with data Mth1 and Std1 become recruited to the *HXT* gene promoters through Rgt1 only in the absence of glucose (Mosley *et al.* 2003). However, the association of these proteins to *HXT* gene promoters is independent of carbon source in *grr1Δ* mutant, which shows constitutive repression of *HXT* expression (Ozcan and Johnston 1995; Ozcan *et al.* 1996a).

In a strain lacking both *STD1* and *MTH1* repression of *HXT* gene transcription by Rgt1 in absence of glucose is completely abolished (Schmidt *et al* 1999; Lafuente *et al.* 2000; Flick *et al.* 2003). This is because Rgt1 is always phosphorylated in the *std1Δmth1 Δ* mutant which relieves Rgt1 from *HXT* gene promoters and inducing *HXT* expression. Mth1 and Std1 are degraded in presence of glucose and this degradation is dependent on casein kinase 1 proteins, Yck1 and Yck2 (Moriya and Johnston 2004) and also Grr1 (Flick *et al.* 2003). Yck1 and Yck2 phosphorylate Mth1 and Std1 at consensus target sequence SXXS (Moriya and Johnston 2004). The conserved regions of Mth1 (residues 118 to 136) and Std1 (residues 129 to 147) contain several matches of the SXXS (Moriya and Johnston 2004). Phosphorylated Mth1 and Std1 are degraded via a Grr1-dependent mechanism (Johnston and Kim 2005; Moriya and Johnston 2004). Mth1 and Std1 have 19 conserved lysine residues and in my work, I show the requirement of the lysine residues for the ubiquitination.

```
Mth1    1 MFVSPPPATSKNQVLQRRPLE---------STNSNHGFASSLQAIPENTMSGSDNASFQS
Std1    1 MFVSPPPATARNQVLGKRKSKRHDENPKNVQPNADTEMTNSVPSIGFNSNLPHNNQEINT

Mth1   52 LPLSMSSSQSTTSSRRENFVNAPPEYTDRARDEIKKRLLASSPSRRSH-HSSSMHSAS-R
Std1   61 PNHYNLSSNSGNVRSNNNFVTTPPEYADRARIEIIKRLLPTAGTKPMEVNSNTAENANIQ

Mth1  110 RSSVAESGSLLSDNASSYQSSIFSAPSTVHTQLTNDSSFSEFPNHKLITRVSLDEALPKT
Std1  121 HINTPDSQSFVSDHSSSYESSIFSQPSTALTDITTGSSLIDTKTPKFVTEVTLEDALPKT

Mth1  170 FYDMYSPDILLADPSNILCNGRPKFTKRELLDWDLNDIRSLLIVEKLRPEWGNQLPEVIT
Std1  181 FYDMYSPEVLMSDPANILYNGRPKFTKRELLDWDLNDIRSLLIVEQLRPEWGSQLPTVVT

Mth1  230 VGDNMPQFRLQLLPLYSSDETIIATLVHSDLYMEANLDYEFKLTSAKYTVATARKRHEHI
Std1  241 SGINLPQFRLQLLPLSSSDEFIIATLVNSDLYIEANLDRNFKLTSAKYTVASARKRHEEM

Mth1  290 TGRNEAVMNLSKPEWRNIIENYLLNIAVEAQCRFDFKQRCSEYKKWKLQQSNLKRPDMPP
Std1  301 TGSKEPIMRLSKPEWRNIIENYLLNVAVEAQCRYDFKQKRSEYKRWKLLNSNLKRPDMPP

Mth1  350 PSIIPR----KNSTETKSLLKKALLKNIQLKNPNNNLDELMMRSSAATNQQGKNKVSLSK
Std1  361 PSLIPHGFKIHDCTNSGSLLKKALMKNLQLKNYKNDAKTL----GAGTQKNVVNKVSLTS

Mth1  406 EEKATIWSQCQAQVYQRLGLDWQPDSVS
Std1  417 EERAAIWFQCQTQVYQRLGLDWKPDGMS
```

Figure 4. Amino acid sequence alignment of Mth1 and Std1 proteins. Protein sequences are taken from *Saccharomyces genome database* (SGD). Sequences are aligned using SIM alignment tool software. Residues identical in two proteins are shown in color.

Glucose Signaling in Yeast and Mammalian Systems

Glucose induction of glucose transporters in yeast is reminiscent of glucose induction of glucose transporters in mammalian cells. Glucose increases the number of glucose transporters in both yeast and mammalian cells (Ozcan and Johnston 1999). In yeast this is achieved by directly increasing the expression of the *HXT* genes, but in mammalian cells it is accomplished indirectly through the action of hormone insulin, which stimulates insertion of the Glut4 glucose transporter into the plasma membrane of fat and muscle cells (Mueckler 1994). Insulin-producing β-cells of pancreas are mostly involved in measuring concentration of glucose in the blood. Glucose increases the amount of insulin secreted by these cells in at least two ways, 1) it stimulates secretion of

insulin that is stored in vesicles and 2) increases insulin gene transcription. These events require glucose transporter Glut2 and glucokinase (Newgard and McGarry 1995). The signal for stimulation of insulin secretion is ATP that is generated from glucose metabolism (Ozcan and Johnston 1999). Glucokinase serves as the glucose sensor as it catalyses the rate limiting step of glucose metabolism in β-cells (Efrat *et al.* 1994).

Two plasma membrane proteins of mammalian cells, GLUT2 and SGLT3 act as glucose sensors, but their mode of action is unrelated to those of Snf3/Rgt2 or Gpr1. GLUT2, a low affinity glucose transporter, appears to work mainly through its control of the glycolytic flux in liver cells (Antoine *et al.* 1997), but not in pancreatic β cells (Efrat *et al.* 1994). SGLT3 protein belongs to sodium/glucose co-transporter family. In presence of glucose it causes a depolarization of plasma membrane because of Na^{+}/glucose transport or of the activation of an ion channel sensitive to glucose (Diez-Sampedro *et al.* 2003). The depolarization triggers an increase in intracellular Ca^{++}, that in the case of pancreatic β cells stimulates insulin secretion (Tarasov *et al.* 2004) and in the case of glucose-sensing neurons, inhibits glucagon secretion from pancreatic α cells, mediated by autonomous nervous system (Gromada *et al.* 2007).

Saccharomyces cerevisiae Unusual Glucose Metabolism

S. cerevisiae has a unique lifestyle, it prefers to ferment glucose rather than oxidize glucose, even when oxygen is present in abundance (Lagunas 1979; Lagunas 1986). Glucose is metabolized through glycolysis to pyruvate. Depending on the availability of oxygen, the fate of pyruvate differs. In presence of oxygen most organisms convert pyruvate into carbon dioxide and water generating many ATPs (36 ATPs per

glucose molecule used). Only when oxygen is limiting, most cells resort to fermentation which yields only 2 ATPs per molecule of glucose via substrate-level phosphorylation of ADP. *S. cerevisiae* prefers to ferment even when oxygen is abundant, which demands them to aggressively utilize the available carbon at the expense of their more efficient competitors (Pfeiffer *et al.* 2001). This unique lifestyle of yeast (aerobic fermentation) is called the 'Crabtree effect' named after the oncologist who discovered this phenomenon in mammalian tumor cells in 1920s (Crabtree 1929). The anoxic, glucose-limited conditions of tumor cells, requires the induction of expression of genes critical for their survival (Johnston and Kim 2005). These include the gene encoding vascular endothelial growth factor (VEGF), which stimulates blood vessel growth, and genes encoding for glycolytic enzymes and low-affinity, high capacity glucose transporter Glut1 (required for aerobic-fermentation lifestyle, to provide high amount of glucose). Expression of these genes is regulated by Hif1 (*h*ypoxia-*i*nduced transcription *f*actor) (Semenza 1998; Chen *et al.* 2001; Seagroves *et al.* 2001), which is activated when oxygen levels decrease, as they do for most cells in a growing tumor. Interestingly, Hif1 function is, like Rgt1, is regulated by its ubiquitination catalysed by an SCF ubiquitin-protein ligase similar to SCF^{Grr1} (Ivan *et al.* 2001; Jaakkola *et al.* 2001). The SCF^{VHL}, one component of which is encoded by the von Hippel-landau tumor suppressor gene, operates on Hif1. Because of similarities between yeast cells and tumor cells, a deeper understanding of mechanisms responsible for glucose induction of gene expression in yeasts will inform cancer biology. This information may open opportunities for developing therapeutic interventions.

CHAPTER II

INTRODUCTION

The budding yeast *Saccharomyces cerevisiae* prefers to ferment glucose even when oxygen is abundant (Lagunas 1979; Lagunas 1986; Pfeiffer 2001). This unusual lifestyle (aerobic fermentation) yields only two ATPs per molecule of glucose fermented, requiring yeast cells to pump large amounts of glucose through glycolysis. This is achieved by enhancing the rate-limiting step of glucose metabolism, its transport into cells by increasing expression of *HXT* genes encoding for glucose transporters. Glucose induces the expression of *HXT* genes via Snf3/Rgt2-Rgt1 signal transduction pathway in which the glucose signal generated by the Snf3 and Rgt2 glucose sensors ultimately alters function of Rgt1 transcription factor (Ozcan *et al.* 1996; Ozcan and Johnston 1999; Forsberg and Ljungdahl 2001).

In the absence of glucose, Rgt1 a DNA binding transcription factor represses the expression of *HXT* genes in conjunction with Mth1, Std1 and general transcription factors Ssn6 and Tup1 (Tomas-Cobos 2002 and Sanz; Lakshmanan *et al.* 2003; Polish *et al.* 2005). Glucose disrupts this interaction by promoting the degradation of Mth1 and Std1 (Flick *et al.* 2003; Moriya and Johnston 2004; Kim *et al.* 2006), thereby relieving the repression of *HXT* expression (Flick *et al.* 2003; Mosley *et al.* 2003; Kim *et al.* 2003).

Mth1 and Std1 are subject to phosphorylation-driven ubiquitination and subsequent degradation when glucose levels are high. According to a current working model, glucose binding to glucose sensors activates the Yck1/2 kinases, which phosphorylate Mth1 and Std1 (Moriya and Johnston 2004). Phosphorylated Mth1 and Std1 are ubiquitinated by the SCF^{Grr1} ubiquitin protein ligase, targeting them for

degradation by the 26S proteasome (Flick *et al.* 2003; Kim *et al.* 2006). Dominant mutations in the glucose sensor genes, *RGT2-1* (Arg231 to Lys) and *SNF3-1* (Arg-229 to Lys) confer the glucose-independent induction of *HXT* expression (Ozcan *et al.* 1996; Ozcan *et al.* 1998). This finding suggests that glucose transport is not required for generation of signal; rather glucose directly binds and activates the glucose sensors, which initiate receptor-mediated signaling (Johnston and Kim 2005). However it has not been demonstrated whether *RGT2-1* and *SNF3-1* cause induction of *HXT* expression by promoting degradation of Mth1 and Std1. In chapter IV, I show that *RGT2-1* promotes degradation of Mth1 and Std1 independent of the presence of glucose. This supports the view that *RGT2-1* locks the protein in the glucose-bound conformation, thus causing constitutive activation of glucose sensor signaling pathway (Ozcan *et al.* 1996). Ubiquitin molecules are added to the lysine residues of the substrate proteins and are targeted to degradation by 26S proteasome. I have mutated lysine residues individually and simultaneously in Mth1 and Std1 to identify the lysine residues responsible for the ubiquitination of these proteins and showed that the evolutionary conserved lysine sites serve as attachment sites for both glucose promoted and glucose independent (*RGT2-1*) degradation of Mth1 and Std1.

The plasma membrane localized Yck1/2 are responsible for the phosphorylation of Mth1 and Std1. Yck1/2 phosphorylate Mth1 and Std1 *in vitro*, altering the serine clusters in the Yck1/2 phosphorylation consensus sites in Mth1 and Std1 prevents degradation of the proteins (Moriya and Johnston 2004). It has not been clearly shown how the Rgt2/Snf3 pathway is activated by glucose. According to the current hypothesis, the glucose sensors undergo glucose-induced conformational change, which probably

activate Yck1/2 by an unknown mechanism that enables them to catalyze the phosphorylation of Mth1 and Std1 (Moriya and Johnston 2004). This idea has been supported by early studies that Mth1 and Std1 show two-hybrid interaction with the C-terminal tails of the glucose sensors (Lakshmanan *et al.* 2003; Schmidt *et al.* 1999; Lafuente *et al.* 2000), which probably places Mth1 and Std1 in proximity to the Yck1/2 protein kinases (Moriya and Johnston 2004). Therefore, coupling of Yck1/2 to the glucose sensors has been hypothesized as a crucial regulatory step that activates the pathway. It indeed, has been thought that Mth1 and Std1 are recruited to the vicinity of Yck1/2 when glucose levels are high (Johnston and Kim 2005; Moriya and Johnston 2004). Yck2 has been shown to be associated with the plasma membrane through the C-terminal Cys-Cys sequence that is palmitoylated in a palmitoyl transferase, Akr1-dependent manner (Feng and Davis 2000). The mammalian casein kinase CK1 isoforms are known to be constitutively active, but the activity of the isoforms appear to be influenced by different mechanisms including subcellular localization, inhibitory autophosphorylation and proteolytic cleavage of C-terminal domain (Knippschild *et al.* 2005). However, mechanisms underlying activation of Yck1/2 have not been clearly elucidated. I tested the aforementioned hypothesis and the results suggest that membrane tethering of Yck1/2 is not absolutely required for the glucose induced degradation of Mth1 and Std1. Mth1 and Std1 are present in the nucleus in the absence of glucose and it is thought that glucose promotes nuclear exclusion of these proteins subjecting them to phosphorylation and ultimately degradation. I have looked at the localization of Mth1 and Std1 and the compartment in which they are degraded in the presence of glucose.

Rgt1 represses hexose transporter genes in the absence of glucose and this repression by Rgt1 requires the presence of Mth1 and Std1. Identifying target genes (other than *HXT* genes) may provide important clues about other unidentified roles of Mth1 and Std1. I have screened for the target genes of Mth1 and Std1 by microarray analysis of *mth1Δstd1Δ* mutants and identified different functional categories of genes regulated by Mth1 and Std1. The newly identified target genes included genes with and without Rgt1-binding consensus sites.

CHAPTER III

MATERIALS AND METHODS

Yeast Strains and Plasmids

Table. 1 Yeast strains used in this study.

Strain	***Genotype***	***Reference***
YM4127	*MATa ura3-52 his3-200 ade2-101 lys2-801 leu2 trp1- 903 tyr1-501 MET GAL4 GAL80*	(Ozcan *et al.*1996)
YM4825	*MATa ura3 his3 ADE2 lys2 leu2 TRP1 RGT2-1 grr1Δ::hisG- URA3-hisG*	(Ozcan *et al.*1996)
BY4741	*MATa his3Δ1 leu2Δ0 ura3Δ0 met15Δ0*	(Ozcan *et al.*1996)
YM6545	*MATa his3Δ1 leu2Δ0 ura3Δ0 met15Δ0RGT2-1*	(Kaniak *et al.* 2004)
YM6548	*MATa his3Δ1 leu2Δ0 ura3Δ0 met15Δ0SNF3-1*	(Kaniak *et al.* 2004)
YM6292	*MATahis3Δ leu2Δ ura3Δmet15Δstd1::KanMX mth1::kanMX*	(Kim *et al.* 2006)
JKY1	YM6548*pdr5::NAT*	This study
JKY20	YM6545*pdr5::NAT*	This study
JKY30	YM4825*pdr5::NAT*	This study
JKY55	*MATa his3Δ1 leu2Δ0 ura3Δ0 met15Δ0 akr1::NAT*	This study
std1Δ	*MATa his3Δ1 leu2Δ0 ura3Δ0 met15Δ0 std1::KanMX*	(Winzeler *et al.* 1999)
mth1Δ	*MATa his3Δ1 leu2Δ0 ura3Δ0 met15Δ0 mth1::KanMX*	(Winzeler *et al.* 1999)
KY33	*MATa his3Δ1 leu2Δ0 ura3Δ0 met15Δ0 mth1::KanMx Std1::NAT*	This study

Table. 2 List of the plasmids used in this study.

Plasmid	*Characteristics*	*Reference*
pBM4560	Mth1-myc	(Moriya and Johnston 2004)
pBM4560	Std1-myc	(Moriya and Johnston 2004)
JHB151	GFP-Mth1	(Kim *et al.* 2006)
JHB149	GFP-Std1	(Kim *et al.* 2006)
pUG36	*ARSH4/CEN-URA3-MET25* promoter-yeGFP-polylinker-*CYC1-terminator*	(Kim *et al.* 2006)
KP51	pUG36-GFP-Mth1-3KA	This Study
KP52	pUG36-GFP-Mth1-5KA	This Study
KP53	pUG36-GFP-MTH1-K86,87A	This Study
KP54	pUG36-GFP-MTH1-K168A	This Study
KP55	pUG36-GFP-MTH1-K193,6A	This Study
KP56	pUG36-GFP-MTH1-K276A	This Study
KP57	pUG36-GFP-MTH1-K301A	This Study
KP58	pUG36-GFP-MTH1-K326A	This Study
KP59	pUG36-GFP-MTH1-K400A	This Study
KP60	pUG36-GFP-MTH1-K405,8A	This Study
KP61	pUG36-GFP-MTH1-K271A	This Study
KP62	pUG36-GFP-MTH1-K343A	This Study
KP63	pUG36-GFP-MTH1-K366A	This Study
KP64	pUG36-GFP-MTH1-K 371 A	This Study
KP65	pUG36-GFP-MTH1-K376A	This Study
KP67	pUG36-GFP-Std1-K17,21A	This Study
KP68	pUG36-GFP-Std1-K96A	This Study
KP69	pUG36-GFP-Std1-K105A	This Study

KP70	pUG36-GFP-Std1-K166A	This Study
KP71	pUG36-GFP-Std1-K179A	This Study
KP72	pUG36-GFP-Std1-K207A	This Study
KP73	pUG36-GFP-Std1-K281A	This Study
KP74	pUG36-GFP-Std1-K287A	This Study
KP75	pUG36-GFP-Std1-K312A	This Study
KP76	pUG36-GFP-Std1-K337A	This Study
KP77	pUG36-GFP-Std1-K344,47A	This Study
KP78	pUG36-GFP-Std1-K 354A	This Study
KP79	pUG36-GFP-Std1-K380,81A	This Study
KP80	pUG36-GFP-Std1-K385A	This Study
KP81	pUG36-GFP-Std1-K390A	This Study
KP82	pUG36-GFP-Std1-K411A	This Study
KP90	pUG36-GFP-Mth1-Δ118-138	This Study
KP91	pUG36-GFP-Std1-Δ129-148	This Study
KP10	NES-GFP-MTH1	This Study
KP11	NES (m)-GFP-MTH1	This Study
KP8	NLS-GFP-MTH1	This Study
KP9	NLS (m)-GFP-MTH1	This Study

Yeast strains were grown on YPD (1% w/v yeast extract, 2% w/v bacto-peptone, and 4% w/v glucose) or synthetic yeast nitrogen base media (0.17% w/v yeast nitrogen base with 0.5% w/v ammonium sulphate) supplemented with appropriate amino acids and carbon sources. All the strains were grown at 30°C. yck^{ts} (*yck1Δyck2^{ts}*) which are temperature sensitive and lose the Yck1/2 function at higher temperature (37°C) were grown at 23°C and at mid-log phase were shifted to 37°C for 30 minutes. All media and

basic yeast methods, such as lithium acetate transformation, were done according to the standard procedures (Sherman 2002). Genes were disrupted by homologous recombination using KanMX (Wach *et al.* 1994) or NatMX cassettes (Goldstein *et al.* 1999).

Plasmids expressing the mutant Mth1 and Std1 proteins were generated using gap repair method (Wach *et al.* 1994; Ma *et al.* 1987) and subcloning protocols. Briefly two oligonucleotides carrying complementary nucleotide changes that result in a single nucleotide substitution were used as primers along with the oligonucleotides flanking *MTH1* or *STD1* to amplify the 5' and 3' portions of the genes in separate reactions, using pBM4748 (*MTH1*) or pBM4747 (*STD1*) (Kim *et al.* 2006) as a template. The *mth1Δstd1Δ* strain (YM6292) was co-transformed with the PCR products and the plasmid pUG34 or pUG36 (Kim *et al.* 2006) cut with BamH1. All the mutations were confirmed by sequencing (Seq Wright, TX). To construct the Mth1 tagged with either wild-type nuclear export signal (NES, ELALKLAGLDIN) or mutant nuclear export signal (NESm, ELALKLAGADIN) leucine-rich nuclear export sequences of PKIα (Feng *et al.* 1999), synthetic oligonucleotides encoding the NES and NESm peptides were fused in frame to the N-terminus of *MTH1*. The NLS peptides of wild-type (NLS, CTPPKKKRKV) or mutant (NLSm, CTPPKTKRKV) of SV40 large T antigen were also in frame fused to the 5' end of the *MTH1* gene.

Fluorescence Microscopy

GFP-fusion proteins expressed in yeast cells were visualized using a Zeiss LSM 510 META confocal laser scanning microscope with a 63x Plan-Apochromat 1.4 NA Oil

DIC objective lens (Zeiss). All images documenting GFP localization were acquired with Zeiss LSM 510 software version 3.2. For microscopy studies all the cells were grown in synthetic yeast nitrogen base media (0.17% yeast nitrogen base with 0.5% ammonium sulphate) supplemented with appropriate amino acids and carbon sources. For FRAP of GFP-Mth1 localized to two specific foci within the nucleus, one of the foci was bleached with a laser pulse at t = 20 s lasting between 0.1 and 0.5 s at 100% power without scanning. Fluorescence recovery was determined every 20 s thereafter for the remainder of the experiment as described previously (Menon *et al.* 2005). For time-lapse microscopy, cells expressing GFP-Mth1 were grown in synthetic yeast nitrogen base media (SYNB) supplemented with appropriate amino acids and 2% galactose were grown to mid-log phase and aliquots of these cells were placed on glass slide with agarose pad (agarose pads were made with SYNB + 4% glucose + 0.1 % electrophoresis grade agarose) and images were taken immediately every minute for a total of 30 minutes.

Western Blotting and Immunoprecipitation (IP)

Western blotting was performed as described previously (Kim *et al.* 2003). Briefly, 5 ml of yeast cells ($O.D_{600}$ = 1.2) were collected by centrifugation at 3,000 rpm in a table-top centrifuge for 5 min. The cell pellets were resuspended in 100 µl of SDS-buffer (50 mM Tris-HCl pH 6.8, 10% glycerol, 2% SDS and 5% β-mercaptoethanol) and boiled for 5 min. After the lysates were cleared by centrifugation at 12,000 rpm for 10 min., soluble proteins were resolved by SDS-PAGE and transferred to PVDF membrane (Millipore). The membranes were incubated with appropriate antibodies in TBST buffer (10 mM Tris-HCl pH 7.5, 150 mM NaCl and 0.05% Tween-20) and proteins were

detected by the enhanced chemiluminescence (ECL) system (Pierce). For IP, yeast lysates were incubated with appropriate antibodies at 4°C for 3 h and further incubated with protein A/G-conjugated agarose beads (Santa Cruz) for 1h (Kim *et al.* 2003).

β-galactosidase Assay

To assay β-galactosidase activity with yeast cells expressing appropriate lacZ reporters, the yeast cells were grown to mid-log phase and assay was performed as described previously (Kaniak *et al.* 2004). Results were reported in Miller Units [(1,000 x OD_{420})/(T x V x OD_{600}), where OD_{420} was the optical density at 420 nm, T was the incubation time in minutes, and V is the volume of cells in milliliters]. The reported enzyme activities were averages of the results from triplicates of three different transformants.

In vitro Phosphorylation Assay

To affinity purify Yck1, extracts of yeast cells expressing the Yck1-His-ProA (Moriya and Johnston 2004) were prepared by vortexing cells with acid-washed glass beads (0.5-mm diameter) in NP-40 lysis buffer (50 mM Tris-HCl pH 8.0, 150 mM NaCl and 1% NP-40) containing phosphatase inhibitors (10 mM Na-pyrophosphatase, 200 μM Na-orthovanadate, 50 mM Na-fluoride) at 4°C for 10 min. The cell lysates were incubated with the anti-ProA antibody conjugated to agarose beads (Santa Cruz Biotechnology) in the NP-40 buffer. After washing with NP-40 buffer containing 1 M NaCl, the Yck1-His-ProA beads were equilibrated with kinase buffer (50 mM Tris-Cl pH 6.8, 150 mM NaCl, 0.5% Triton X-100 and 1mM dithiothreitol). Gst-Mth1 expressed in

E .coli was also affinity purified with glutathione-Sepharose-4B beads (Amersham Biosciences). The Yck1-His-ProA and Gst-Mth1 were mixed in 50 µl of the kinase buffer containing 0.5 mCi of [γ^{32}P] ATP, 100 µM ATP, 10 mM $MgCl_2$ and incubated at 28°C for 30 min. After washing the beads with kinase buffer containing 0.5 M NaCl, the proteins were eluted by boiling the beads in SDS-sample buffer for 5 min. The eluted proteins were resolved by SDS-PAGE and detected by autoradiography.

Mth1 and Std1 Protein Alignment and ClustalW Protein Alignment

Amino acid sequence alignment of Mth1 and Std1 proteins was done using SIM alignment tool software. Protein sequences were taken from *Saccharomyces genome database* (SGD) and aligned using SIM alignment tool software. Homology search of orthologs of Mth1 and Std1 from other yeast species was done using the Clustal W protein alignment (Chenna *et al.* 2003) which is provided in *Saccharomyces* genome database.

Transcriptomic Analysis

Microarray analyses were done by isolating total RNA (Wang et al. 2004) from isogenic *mth1Δstd1Δ* and wild type cultures grown to early logarithmic phase in YEP containing 2% galactose as the carbon source. For all microarray analysis, the quality of RNA was tested by using an Agilent bioanalyzer 2100 with RNA Nano 6000 Labchips. Samples were labeled with Cy3-CTP or Cy5-CTP by using a low input fluorescent linear amplification kit (Agilent Technologies). Labeled cRNA was purified with RNeasy MinElute kit (Qiagen) and hybridized to yeast 60-mer oligonucleotide arrays (Agilent

Technologies) according to the manufacturer's instructions. Slides were scanned at 10 μm resolution with 2-line averaging using an Axon GenePix 4200A scanner and GenePix software. Ratio-based and LOWESS normalization as well as statistical analysis were done in Acuity 4.0 (Molecular Dynamics). Misregulated genes in *mth1Δstd1Δ* were identified by an average expression change of at least two-fold (i.e a log2 ratio of ≤ -1 or ≥ 1) relative to the isogenic wild type value. Statistical significance of the genes identified by this analysis was confirmed by performing a paired, one-tailed T-test against a control array; genes referred to as statistically significant have a value < 0.001.

Real-Time Quantitative PCR

For real-time quantitative PCR (qPCR) total RNA was isolated (Wang *et al.* 2004) from isogenic *mth1Δstd1Δ*, *mth1Δ*, *std1Δ* and wild-type (FM391) cultures grown to early logarithmic phase in YEP containing 2% galactose as the carbon source. The quality of RNA was tested by using an Agilent bioanalyzer 2100 with RNA Nano 6000 Labchips. The primers used for qPCR were designed with Primer 3 version 0.4.0 software (Massachusetts Institute of Technology) to amplify gene fragments with an optimal size of 80–100 bp. The primer sequences used for qPCR are listed in appendix C. Measurements of relative levels of gene expression were done by qPCR. iScript cDNA synthesis kit (Bio-Rad) was to reverse transcribe RNA into cDNA. The reverse transcriptase reactions were done at 25 °C for 5 min, 42 °C for 30 min and 85 °C for 5 min as per the manufacturer recommendation. cDNA was stored at -20°C until further use. PCR reactions were done in 25 μl reactions by using iQ SYBR Green Supermix (Bio-Rad) as recommended by the manufacturer (Bio-Rad). The reaction mixtures

contained: 5 µl cDNA; 12.5 µl iQ SYBR Green Supermix; 0.5 µl forward primer (1.5 pmol μl^{-1}); 0.5 µl reverse primer (1.5 pmol μl^{-1}); and 6.5 µl de-ionized H_2O (Sambanthamoorthy *et al.* 2006). All qPCR reactions were done in triplicate and the mean C_T was used for analysis of results. To verify the absence of contaminating DNA, each qPCR experiment included controls that lacked template cDNA. The constitutively expressed gene for actin (*ACT1*) was used as an endogenous control as described previously (Brickner *et al.* 2007). Analysis of expression of each gene was done based on at least two independent experiments.

CHAPTER IV

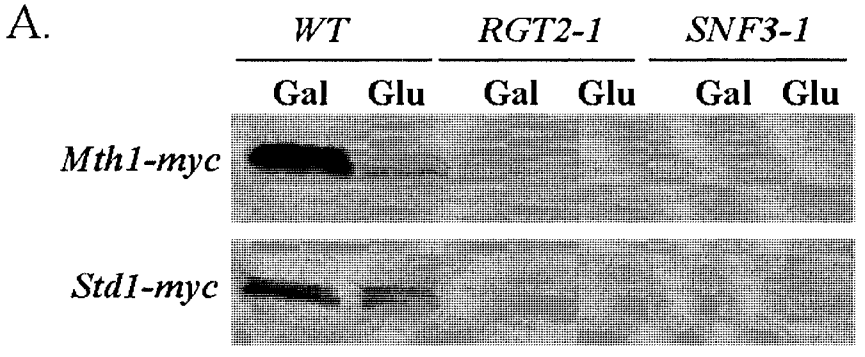
BIOCHEMICAL EVIDENCE FOR GLUCOSE-INDEPENDENT INDUCTION OF *HXT* EXPRESSION IN *SACCHAROMYCES CEREVISIAE*

The budding yeast *S. cerevisiae* growing on high levels of glucose induces *HXT* expression, which facilitates the rate limiting step of glucose utilization – glucose uptake. This is achieved by derepressing the Rgt1-repressed *HXT* expression via the Rgt2/Snf3-Rgt1 signaling pathway (Ozcan and Johnston 1999; Forsberg and Ljungdahl 2000). Dominant mutations in glucose sensor genes, *RGT2-1* (Arg-231 to Lys) and *SNF3-1* (Arg-229 to Lys) confer the glucose-independent induction of *HXT* expression (Ozcan *et al.* 1996; Ozcan *et al.* 1998). This finding suggests that glucose transport is not required for generation of signal; rather glucose directly binds and activates the glucose sensors, which initiate receptor (sensor)-mediated signaling (Johnston and Kim 2005). However, it has not been demonstrated whether *RGT2-1* and *SNF3-1* cause induction of *HXT* expression by promoting the degradation of Mth1 and Std1. In this chapter I show that *RGT2-1* promotes degradation of Mth1 and Std1 independent of the presence of glucose. These results support the view that *RGT2-1* locks the protein in the glucose-bound conformation, and thus causing constitutive activation of the glucose sensor signaling pathway (Ozcan *et al.* 1996).

RGT2-1 and *SNF3-1* Cause Degradation of Mth1 and Std1 Independent of the Presence of Glucose

To address glucose-independent degradation of Mth1 and Std1, I determined cellular levels of Mth1 and Std1 in the *RGT2-1* and *SNF3-1* strains by Western blotting

and confocal microscopy. Mth1-myc and Std1-myc are barely detected by Western blotting in the *RGT2-1* and *SNF3-1* strians grown in medium lacking glucose (Figure. 5A, Gal). To confirm the western data, I looked at the fluorescence levels of GFP-Mth1 and GFP-Std1 using confocal microscope. Fluorescence intensities of GFP-Mth1 and GFP-Std1 are strong in the wild-type cells but are profoundly diminished in the *RGT2-1* and *SNF3-1* strains in the absence of glucose (Figure. 5B, Gal). These results suggest that *RGT2-1* and *SNF3-1* promote degradation of Mth1 and Std1 in a glucose-independent manner. Mth1 degradation is reinforced by glucose repression of *MTH1* expression by Mig1, whereas Std1 degradation is obscured by glucose induction of *STD1* expression through the Rgt2/Snf3-Rgt1 pathway (Figure. 5A, *WT*; Kaniak *et al.* 2004). Indeed *RGT2-1* and *SNF3-1* induce expression of *STD1* gene 3- and 10-fold respectively, in the absence of glucose (Kaniak *et al.* 2004). However, Std1 degradation is accelerated and Mth1 degradation is slowed when glucose regulation of *MTH1* and *STD1* expression is interrupted by replacing their promoters with the *MET25* promoter, which is not regulated by glucose (Kim *et al.* 2006).



B.

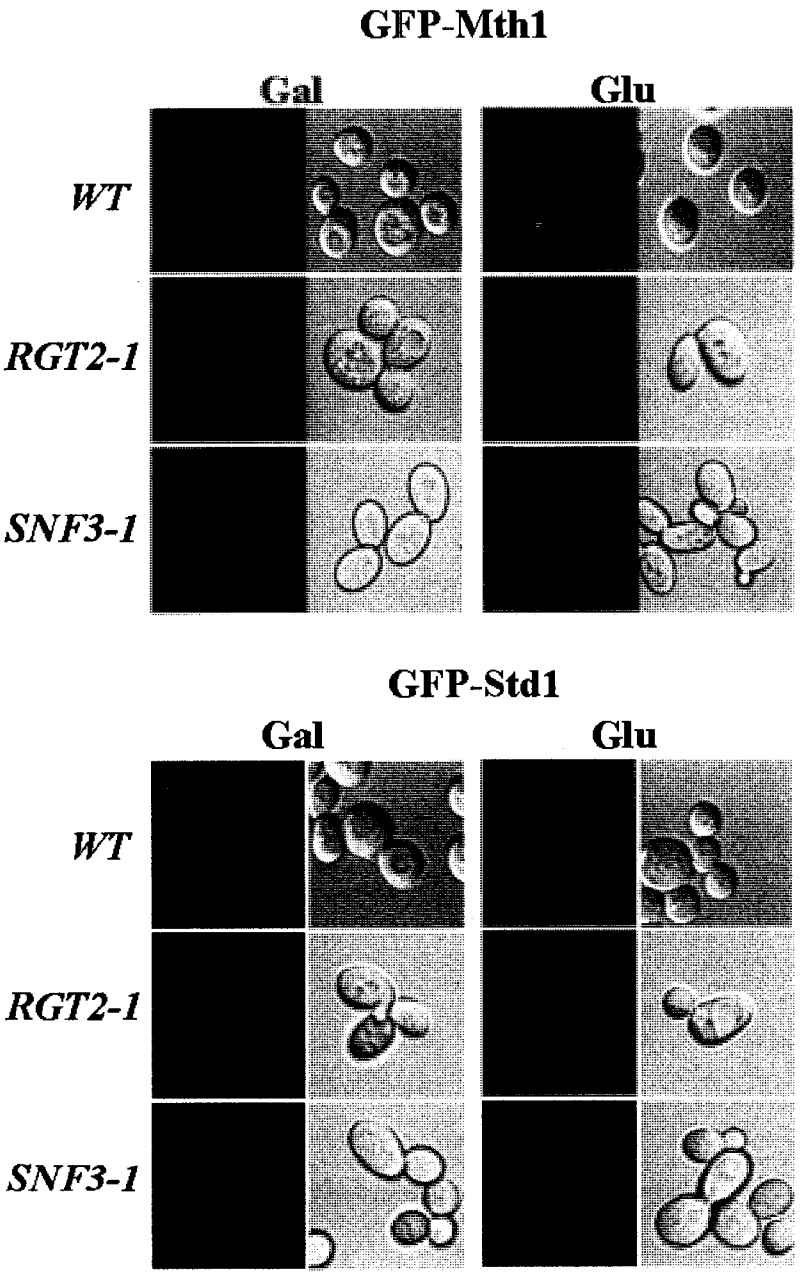


Figure 5. *RGT2-1* and *SNF3-1* promote glucose-independent degradation of Mth1 and Std1. (A) Yeast cells expressing Mth1-myc or Std1-myc under the control of their own promoters (Moriya and Johnston 2004) were grown to mid-log phase in a selective medium containing 2% galactose. Aliquots were then transferred to 2% galactose medium (Gal) or 4% glucose medium (Glu) and incubated for 60 min. Levels of Mth1-myc and Std1-myc were determined by Western blotting using anti-myc antibody. (B) Yeast cells expressing GFP-Mth1 or GFP-Std1 under the control of *MET25* promoter

(Kim *et al.* 2006) were grown as described above. Cells were observed under the Zeiss LSM 510 META confocal laser scanning microscope. DIC and GFP fluorescence images are shown.

Glucose-Independent Degradation of Mth1 and Std1 Requires the SCF^{Grr1}-26S Proteasome Pathway

Normal glucose-induced degradation of Mth1 and Std1 requires SCF^{Grr1} and 26S proteasome (Flick et al. 2003; Kim et al. 2006). We observe that degradation of Mth1 and Std1 in the *RGT2-1* or *SNF3-1* strains is impaired when *GRR1* is deleted (Figure. 6, *RGT2-1grr1Δ* or *SNF3-1grr1Δ*) or when yeast cells are treated with MG132, a chemical inhibitor of the 26S proteasome (Figure. 6, *RGT2-1pdr5Δ* + MG132 or *SNF3-1pdr5Δ* + MG132). As MG132 can be extruded from cells via the Pdr5 drug efflux pump, cells lacking the *PDR5* gene were treated with MG132.

Detection of *in vivo* ubiquitination of Mth1 and Std1 appears to be difficult, and which probably is due to unexplained, rapid deubiquitination in high glucose (Kim *et al.* 2006). To detect Ub-Mth1, Spielewoy *et al.* (Spielewoy et al. 2004) took advantage of a mutant form of ubiquitin ($Ubi^{K48R, G76A}$), which generates only monoubiquitinated substrates that are resistant to deubiquitinating enzymes (Willems *et al.* 1996). However, the *in vitro* pull-down experiments were carried out with Mth1 expressed from the galactose-inducible *GAL* promoter. No glucose signal, however, is likely to be present in the galactose-grown wild-type cells. Therefore, whether Mth1 and Std1 are ubiquitinated *in vivo* has not been conclusively determined. We made use of *RGT2-1* mutant strain which promotes degradation of Mth1 and Std1 even in galactose medium and determined their ubiquitination (Figure. 7).

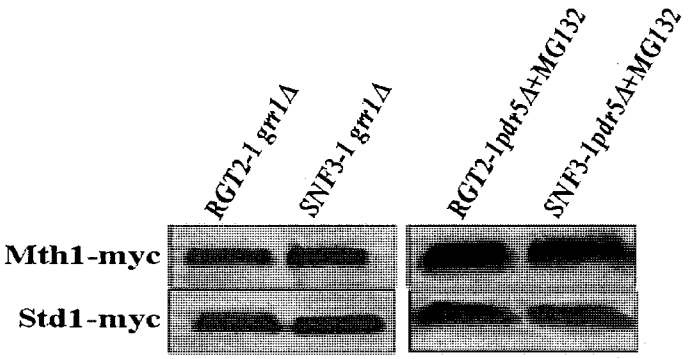


Figure 6. *grr1Δ* and protease inhibitor MG132 abolished the degradation of Mth1-myc and Std1-myc in *RGT2-1* and *SNF3-1*. The *RGT2-1grr1Δ* and *SNF3-1grr1Δ* strains expressing Mth1-myc (Moriya and Johnston 2004) or Std1-myc (Moriya and Johnston 2004) were grown in 2% galactose medium (left panel). The *RGT2-1pdr5Δ* and *SNF3-1pdr5Δ* strains expressing Mth1-myc or Std1-myc were grown in 2% galactose medium and treated with MG132 (50 μg/ml) for 30 min (right panel). Mth1 and Std1 were analyzed by immunoblotting with anti-myc antibody (Santa Cruz).

RGT2-1 Promotes Ubiquitination of Mth1 and Std1 by SCF^{Grr1}

Glucose induces conformational change in glucose sensor proteins Rgt2 and Snf3 which are thought to activate Yck1/2. Activated Yck1/2 phosphorylate paralogous proteins Mth1 and Std1 which are in turn ubiquitinated by SCF^{Grr1} complex and target them to degradation by 26S proteasome. To determine whether *RGT2-1* promotes ubiquitination of Mth1 and Std1 in glucose independent manner, the extracts of *RGT2-1* strain expressing Mth1-myc and Std1-myc, grown in the absence of glucose, were analyzed by immunoprecipitation (IP)-Western blotting (Figure 7). *RGT2-1grr1Δ* strain was used as control because *GRR1* is required to promote ubiquitination of SCF^{Grr1} substrate proteins. In Western blotting of the wild-type cell extracts, anti-myc antibody detects a single band that corresponds to Mth1-myc (Figure 7A, lane 2), whereas anti-Ub antibody is cross reactive to a high-molecular mass ladder, typical of a polyubiquitin chain (Figure 7A,

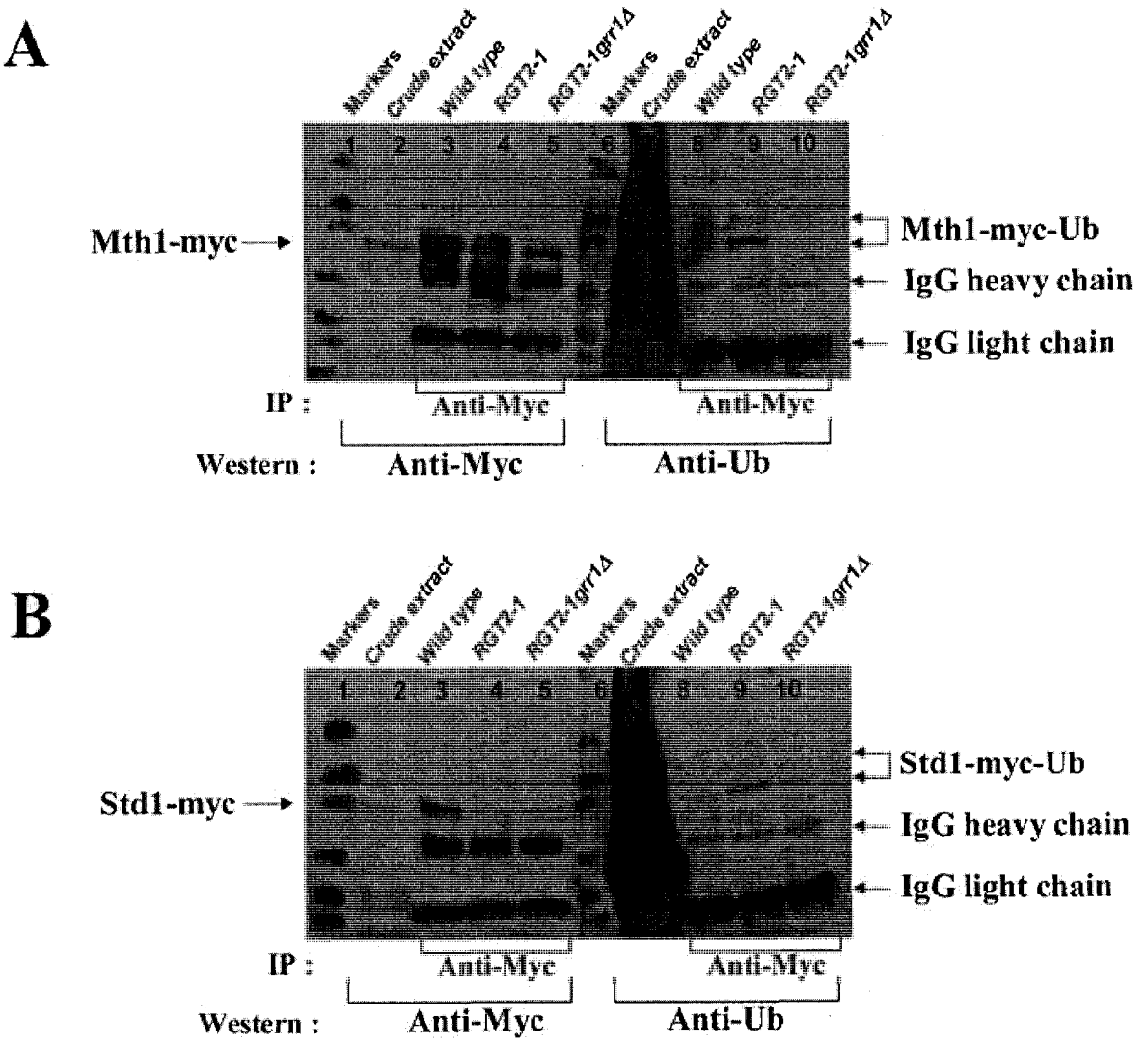


Figure 7. *RGT2-1* promotes ubiquitination of Mth1 and Std1 *in vivo*. The wild-type and *RGT2-1* strains expressing Mth1-myc (A) or Std1-myc (B) were grown in 2% galactose medium. Yeast cell extracts were resolved on an SDS-gel and analyzed by Western using anti-myc antibody (lane 2) and anti-Ub antibody (lane 7). For IP of Mth1-myc and Std1-myc, the proteins were expressed in the wild type (*pdr5Δ*; lanes 3 and 8), *RGT2-1* (*RGT2-1pdr5Δ*; lanes 4 and 9), *RGT2-1grr1Δ* (*RGT2-1grr1Δpdr5Δ*; lanes 5 and 10) strains. For the treatment with the proteasome inhibitor MG132, *PDR5* gene encoding a drug efflux pump was disrupted in yeast stains used. Yeast cells grown in 2% galactose medium were treated with MG132 (50 μg/ml) for 30 min. and disrupted with glass beads in NP-40 buffer to prepare cell lysates (Kim *et al.* 2003). Mth1-myc and Std1-myc in cell lysates were precipitated with anti-myc antibody, resolved on an SDS-gel, and detected by Western blotting using either anti-myc antibody (lanes3-5) or anti-Ub antibody (lanes 8-10)

lane 7). Next, Mth1-myc in cell extracts were precipitated using anti-myc antibody-conjugated beads and then subject to Western blotting using either anti-myc antibody (Figure 7A, lanes 3-5) or anti-Ub antibody (Figure 7A, lanes 8-10). Compared to its wild-type allele (Figure 7A, lane 8), *RGT2-1* greatly enhances Mth1 ubiquitination (Figure 7A, lane 9). However, ubiquitination is largely impaired when *GRR1* is disrupted in the *RGT2-1* strain (*RGT2-1grr1Δ*; Figure 7A, lane 10). Similar observations are also made with Std1 (Figure 7B). Therefore, we concluded that *RGT2-1* promotes ubiquitination of Mth1 and Std1 by SCF^{Grr1} in the absence of glucose.

The Evolutionary Conserved Lysine Residues of Mth1 Are Required for Degradation

The ClustalW protein alignment (*Saccharomyces* Genome Database) shows that Mth1 contains ~ 19 lysines which are well conserved in their orthologs from other yeast species (Figure 8A). The ClustalW protein alignment of Mth1 and Std1 is shown in appendix A. The evolutionary conserved lysines residues in Mth1 are K86, K87, K168, K168, K193, K196, K271, K276, K301, K326, K333, K334, K336, K343, K366, K371, K376, K400, K405 and K408. Individual mutations of 19 conserved lysine residues to alanine did not prevent degradation of Mth1 (data not shown). However, simultaneous mutation of 5 lysines in the carboxy terminal region of Mth1 (5KA; positions K326, K333, K334, K336 and K343) severely impairs degradation of Mth1 (Figure 8B). Mth1-5KA is not degraded in the *RGT2-1* strain.

The Evolutionary Conserved Lysine Residues of Std1 Are Required for Degradation

The ClustalW protein alignment (*Saccharomyces* Genome database) shows that Std1, like Mth1 contains 19 conserved lysine residues. The conserved lysine residues in Std1 are K17, K21, K96, K105, K166, K179, K207, K281, K287, K312, K337, K344, K347, K354, K380, K381, K385, K390 and K411. Individual mutations of the 19 conserved lysine residues in Std1 did not prevent its degradation (data not shown). It's been previously shown that conversion of 9 out of 19 conserved lysines in Std1 to arginine (Std1-9KR) reduces induction of *HXT1* expression by impairing degradation of Std1 (Kim et al. 2006). Like Mth1-5KA, Std1-9KR is not degraded in the *RGT2-1* strain (Figure 8C). These results suggest that the evolutionarily conserved lysine residues might serve as attachment sites for ubiquitin, which is required for both the glucose-promoted and glucose-independent degradation of Mth1 and Std1.

Glucose-Independent Degradation of Mth1 and Std1 Requires the Putative Yck1/2 Phosphorylation Sites.

Yck1/2 appears to phosphorylate Mth1 and Std1 at the conserved cluster of serine residues, known as the Yck1/2 phosphorylation sites [(SXXS) Moriya and Johnston 2004]. The conserved cluster of serine residues in Mth1 include residues- S118, S121, S125, S126, S129, S130, S133 and S136 (Moriya and Johnston 2004). Similarly serine residues- S129, S132, S135, S136, S137, S140, S141, S144 and S147 form part of conserved cluster of serine residues in Std1 (Moriya and Johnston 2004). Deletion of the serine sites in Mth1 (Δ118-138) and Std1 (Δ129-148) prevents both the glucose promoted

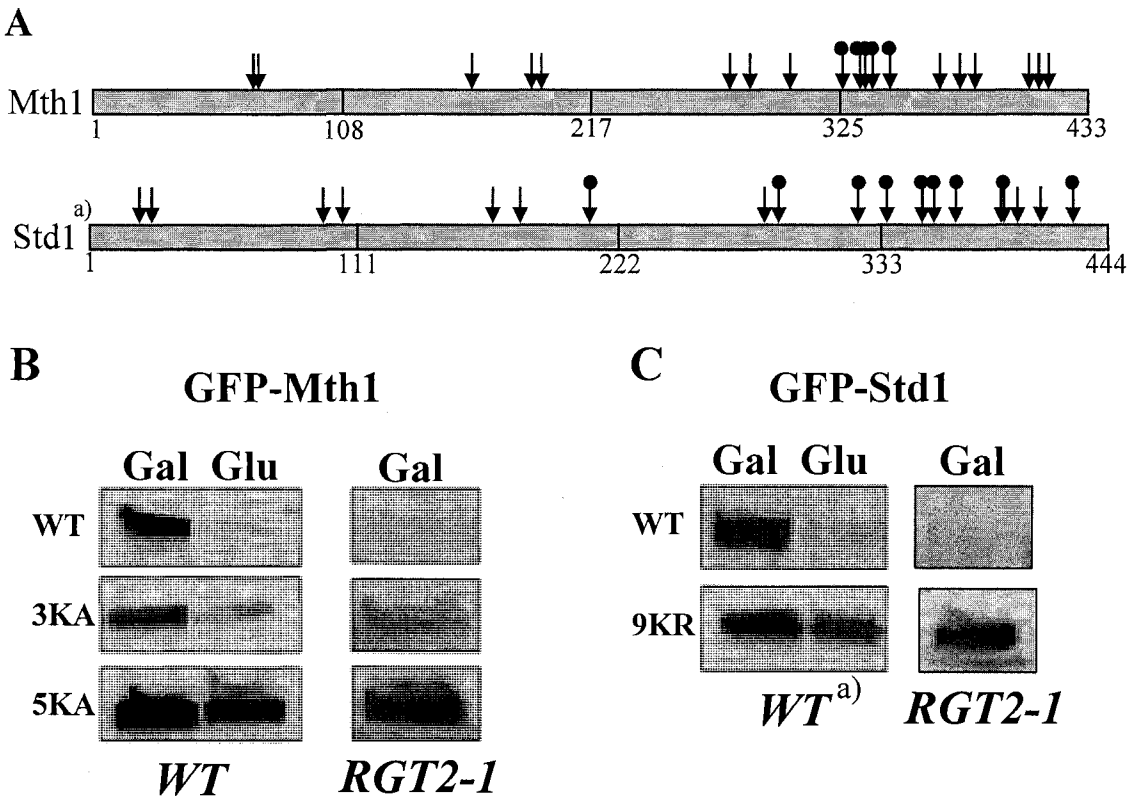


Figure 8. The evolutionary conserved lysine residues of Mth1 and Std1 are required for glucose-independent degradation. (A) Mth1 and Std1 contain ~19 evolutionary conserved lysine residues. Individual mutations of the conserved lysine residues to alanine (arrows) do not prevent degradation of Mth1 and Std1 (data not shown). However, simultaneous mutation of multiple lysine residues in the C-terminal regions of Mth1 and Std1 protects then from degradation (filled circles on arrows). (B and C) The wild-type and *RGT2-1* strains expressing GFP-Mth1 (Kim *et al.* 2006), GFP-Mth1-3KA (KP51), GFP-Mth1-5KA (KP52), GFP-Std1 (Kim *et al.* 2006), and GFP-Std1-9KR (Kim *et al.* 2006) were grown in SYNB containing 2% galactose (Gal) and were shifted SYNB containing 4% glucose for 60 min (Glu). Levels of Mth1 and Std1 were determined by Western blotting using anti-GFP antibody ([a] adapted from Kim *et al.* 2006).

and glucose independent degradation of Mth1 and Std1 (Figure 9). It has been proposed that a conformational change in the glucose sensors upon glucose binding causes activation of Yck1/2 that is tethered to the cell membrane through a C-terminal palmitate moiety in the sequence (Moriya and Johnston 2004). Our results suggest that the *RGT2-1* converts the protein into the glucose-bound form as proposed previously (Ozcan *et al.*

1996), which activates Yck1/2 even in the absence of glucose. Thus Yck1/2 interaction with the glucose sensors appears to be crucial for the activation of the kinases. However, Yck1/2 seems to interact with the glucose sensors in both the presence and absence of glucose (Moriya and Johnston 2004). The molecular mechanism underlying activation of Yck1/2 in response to glucose remains elusive.

The results presented above using dominant glucose sensor mutants *RGT2-1* and *SNF3-1* provide biochemical evidence for the glucose independent expression of *HXT* expression. *RGT2-1* and *SNF3-1* cause induction of *HXT* expression even in the absence of glucose by promoting the degradation of Mth1 and Std1 (Figure 5A).

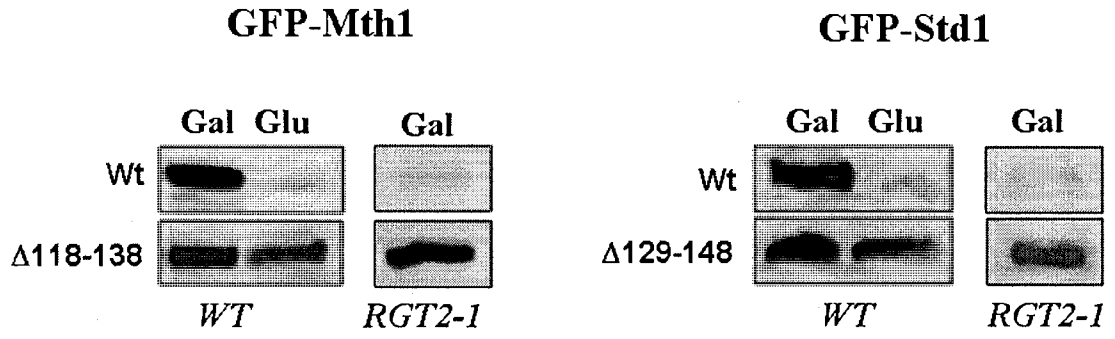


Figure 9. The putative Yck1/2 phosphorylation sites of Mth1 and Std1 are required for glucose –independent degradation of Mth1 and Std1. GFP-Mth1 (Δ118-138; KP90) and GFP-Std1 (Δ129-148; KP91) lacking the Yck1/2 phosphorylation sites were expressed in the wild-type and *RGT2-1* strains. Levels of Mth1 and Std1 were determined by Western blotting using anti-GFP antibody.

Phosphorylation by Yck1/2 (Figure. 9) and ubiquitination by SCF^{Grr1} ubiquitin ligase complex (Figure 7) are required for glucose-independent degradation of Mth1 and Std1.

Role of Snf1 Kinase in the Degradation of Mth1 and std1

The Snf1 kinase plays a crucial role in signaling glucose limitations. Glucose regulates activity and subcellular localization of Snf1 kinase (Vincent *et al.* 2001). Snf1

is active and present in the nucleus upon phosphorylation on threonine 210 when glucose is depleted in the medium (Estruch *et al.* 1992). However, addition of glucose promotes dephosphorylation of Snf1 by the Reg1/Glc7 phosphatase, leading to conversion of the kinase from active to an inactive conformation (Sanz *et al.* 2000). Deletion of *REG1* causes inhibition of *HXT1* expression (Tomas-Cobos and Sanz 2002). In this study, we show that glucose-promoted inactivation of Snf1 is necessary for degradation of Mth1 and Std1.

Glucose-Promoted Inactivation of Snf1 is Necessary for the Degradation of Mth1 and Std1

Removal of the *REG1* gene prevents Mth1 degradation in high glucose (Gadura *et al.* 2006), which may give an explanation of why expression of the *HXT1* gene is constitutively repressed in *reg1Δ* (Tomas-Cobos and Sanz 2002). Snf1 is constitutively active in *reg1Δ*, probably due to a failure in converting the kinase from an active into an inactive conformation (Sanz *et al.* 2000). Therefore, we determined if Snf1 is involved in the stability of Mth1 and Std1 in *reg1Δ* by Western blotting. As seen in Figure 10, considerable amounts of Mth1 are detected in *reg1Δ* grown in high glucose as reported previously (Gadura *et al.* 2006); in contrast, Mth1 is not detected when *SNF1* gene is disrupted in *reg1Δ* (*snf1Δ reg1Δ*) (Figure 10). As aforementioned, glucose not only promotes degradation of Std1, but also induces *STD1* expression via the Rgt2/Snf3-Rgt1 pathway (Kim *et al.* 2006; Kaniak *et al.* 2004). This obscures disappearance of Std1 (Std1-myc in figure 5A and Figure. 10, *WT*, glu). However, Std1 levels are increased by *REG1* deletion (*reg1Δ*) but decreased again by *SNF1* deletion *reg1Δ* (*snf1Δ*

reg1Δ) (Figure 10), suggesting that Std1 degradation is also prevented when Snf1 is not inactivated by glucose. The Sak1 kinase is known to promote activation and nuclear localization of Snf1 upon glucose depletion (Hong *et al.* 2003; Nath *et al.* 2003).

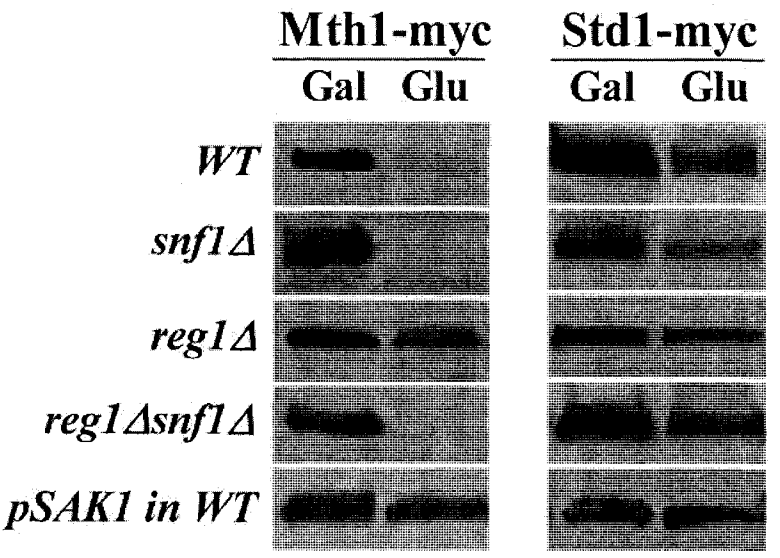


Figure 10. Artificial activation of the Snf1 kinase prevents degradation of Mth1 and Std1. Yeast cells of the indicated genotype expressing Mth1-myc or Std1-myc were grown in 2% galactose medium (Gal) or 4% glucose medium (Glu) as described in Fig. 5. The Sak1 kinase was overexpressed from a high-copy plasmid (2 μ) with Mth1-myc or Std1-myc in wild-type cell *(pSAK1* in *WT*). Levels of Mth1-myc and Std1-myc were determined by Western blotting using anti-myc anti-body. Indeed, overexpression of *SAK1* prevents degradation of Mth1 and Std1 (Figure 10, *pSAK1*). These results suggest that artificially activated Snf1 plays an important role in blocking the glucose-promoted degradation of Mth1 and Std1. In addition, a hyperactive Snf1, Snf1-G53R (Estruch *et al.* 1992), prevents degradation of Mth1 and Std1 in high glucose (Figure 11).

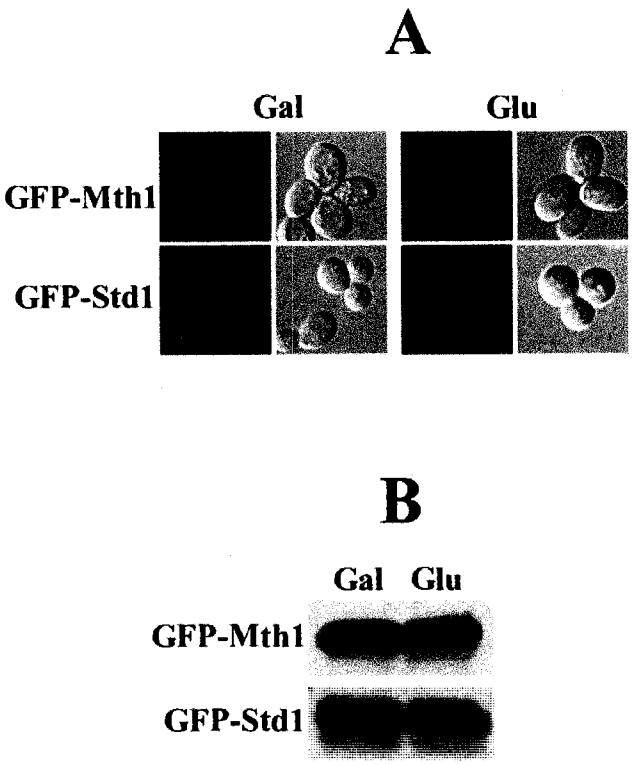


Figure 11. The hyperactive Snf1 kinase prevents degradation of Mth1 and Std1. The hyperactive Snf1 (SNF1-G53R, Estruch *et al.* 1992) was co-expressed with GFP-Mth1 or GFP-Std1 were determined in *snf1Δ*, and levels of GFP-Mth1 and GFP-Std1 were determined by confocal microscopy (A) and Western blotting (B).

It is not known how Snf1 prevents degradation of Mth1 and Std1, when it is not inactivated by high levels of glucose. The proposed model for degradation of Mth1 and Std1 includes nuclear export of the proteins, because they must undergo phosphorylation by the membrane-tethered Yck1/2 prior to being ubiquitinated (Moriya and Johnston 2004). It is possible that Snf1 regulates nuclear export of Mth1 and Std1, because Mth1 and Std1 are found in the nucleus of the cells harboring active Snf1. Snf1 plays a crucial, decisive role in Snf1-Migl signaling that leads to establishment of glucose repression of gene expression. Glucose repression of *SUC2* expression is defective when Mth1 is not degraded (Kim *et al.* 2006; Schulte *et al.* 2000).

Therefore, these observations imply a functional link inactivation of Snf1 and degradation of Mth1 and Std1. This cross-talk may play a key role as a molecular switch that efficiently triggers two functionally distinct glucose signaling pathways- the Rgt2/Snf3-Rgt1 glucose induction pathway and the Snf1-Mig1 glucose repression pathway in response to glucose.

CHAPTER V

SUBCELLUAR LOCALIZATION AND TURNOVER OF PARALOGOUS PROTEINS Mth1 AND Std1

Glucose transport across the cell membrane is the rate-limiting step for its utilization and enhanced by the expression of the glucose transporter genes. The mechanism of glucose induced expression of *HXT* genes is discussed in chapter 1. Glucose induced inactivation of Mth1 and Std1 is probably due to degradation of these proteins in the 26S proteasome through the Rgt2/Snf3 glucose induction pathway that includes the yeast casein kinases Yck1 and Yck2 (Moriya and Johnston 2004), and SCF^{Grr1} (Kim *et al.* 2006; Spielwoy *et al.* 2004). The plasma membrane-localized Yck1/2 are responsible for the phosphorylation of Mth1 and Std1 (Moriya and Johnston 2004). This phosphorylation is crucial for the ubiquitination by SCF^{Grr1} (Kim *et al.* 2006; Pasula *et al.* 2007) and subsequent degradation of Mth1 and Std1.

It has not been clearly shown how the Rgt2/Snf3 pathway is activated by glucose. According to the current hypothesis, the glucose sensors undergo glucose-induced conformational change, which probably activate Yck1/2 by an unknown mechanism that enables them to catalyze phosphorylation of Mth1 and Std1 (Moriya and Johnston 2004). This idea has been supported by the early studies that Mth1 and Std1 show two-hybrid interaction with the C-terminal tails of the glucose sensors (Lakshmanan *et al.* 2003; Schmidt *et al.* 1999; Lafuente *et al.* 2000), which probably places Mth1 and Std1 in proximity to the Yck1/2 protein kinases (Moriya and Johnston 2004). Therefore, coupling of Yck1/2 to the glucose sensors has been hypothesized as a crucial regulatory step that activates the pathway. It, indeed, has been thought that Mth1 and Std1 are recruited to the

vicinity of Yck1/2 when glucose levels are high (Johnston and Kim 2005; Moriya and Johnston 2004).

In the current study, I tested the aforementioned hypothesis and present results that suggest, membrane tethering of Yck1/2 is not absolutely required for the degradation of Mth1 and Std1; however, they are required for induction of *HXT* genes expression. Also discussed in this chapter are the results of localization studies of Mth1 and Std1 and turnover of these proteins (subcellular compartment in which they are degraded) in response to glucose.

Mth1 and Std1 Are Present in the Nucleus when They Are Not Degraded

Mth1 and Std1 are presumed to be present in nucleus in absence of glucose and in conjunction with Rgt1 they repress the expression of *HXT* genes. In presence of glucose Mth1 and Std1 are thought to leave nucleus and get degradaded in cytoplasm. To test, this hypothesis, we first determined the localization of GFP-Mth1 using time-lapse microscopy. To this end, Mth1 fused to GFP at its N-terminus (GFP-Mth1) was expressed under the control of *MET25* promoter (Kim *et al.* 2006) and subcellular localization of the protein was observed by fluorescent microscopy (Figure 12). *WT* cells expressing GFP-Mth1 were grown in galactose medium overnight and followed by time-lapse microscopy after shifting to glucose. About 50% of GFP-Mth1 was degraded within 5-10 min (Figure 12) after glucose was added, which is consistent with the results obtained with Western blot analysis. And moreover this degradation appeared to be taking place in the nucleus all the time. GFP-Mth1 was not degraded in *grr1Δ* cells (Figure 12) even after glucose was added; however, it was not excluded from the nucleus

either. These results suggest that Mth1 (presumably also Std1) does not localize exclusively to cytoplasm in response to glucose (*grr1Δ*); rather they localize to nucleus and are degraded when glucose levels are high.

Figure 12. Time lapse microscopy of Mth1 degradation. Indicated genotype cells expressing GFP-Mth1 were grown in selective media containing 2% galactose to mid-log phase. Galactose grown cells were put on agarose pads made with selective media containing 4% glucose. Images of individual cells were taken every 2 min. for 14 min, using Zeiss LSM 510 confocal microscope.

Mth1 and Std1 are Localized to Nucleus in Mutants where Degradation of Mth1 and Std1 is Prevented

The results above led us to consider whether Mth1 and Std1 are degraded within the nucleus, which is not in harmony with the hypothesis. Thus we addressed whether glucose promotes nuclear export of Mth1 and Std1 by determining subcellular localization of the proteins in circumstances in which glucose-induced degradation of Mth1 and Std1 is prevented, such as disruption of the Rgt2/Snf3 and expression of mutant Mth1 and Std1 proteins resistant to degradation (Figure 13). As observed previously (chapter IV and Pasula *et al.* 2007 and chapter IV, Figure 5B), weak

previously (chapter IV and Pasula *et al.* 2007 and chapter IV, Figure 5B), weak fluorescence signals were detected in the glucose-grown wild type cells (Figure 13) due to the degradation of Mth1 and Std1 (Kim *et al.* 2006; Pasula *et al.* 2007). Importantly, GFP-Mth1 and GFP-Std1 were found in the nucleus of glucose-grown *rgt2Δsnf3Δ* and *grr1Δ* cells (Figure 13). Next, we generated mutations at the putative Yck1/2 phosphorylation sites in Mth1 and Std1 and examined mutant proteins for glucose-dependent degradation and localization. Single amino-acid change in Mth1 (S130A and S133A) was enough to prevent degradation of the protein and the mutant proteins constitutively localized to the nucleus (data not shown). Mth1 in mutant cells

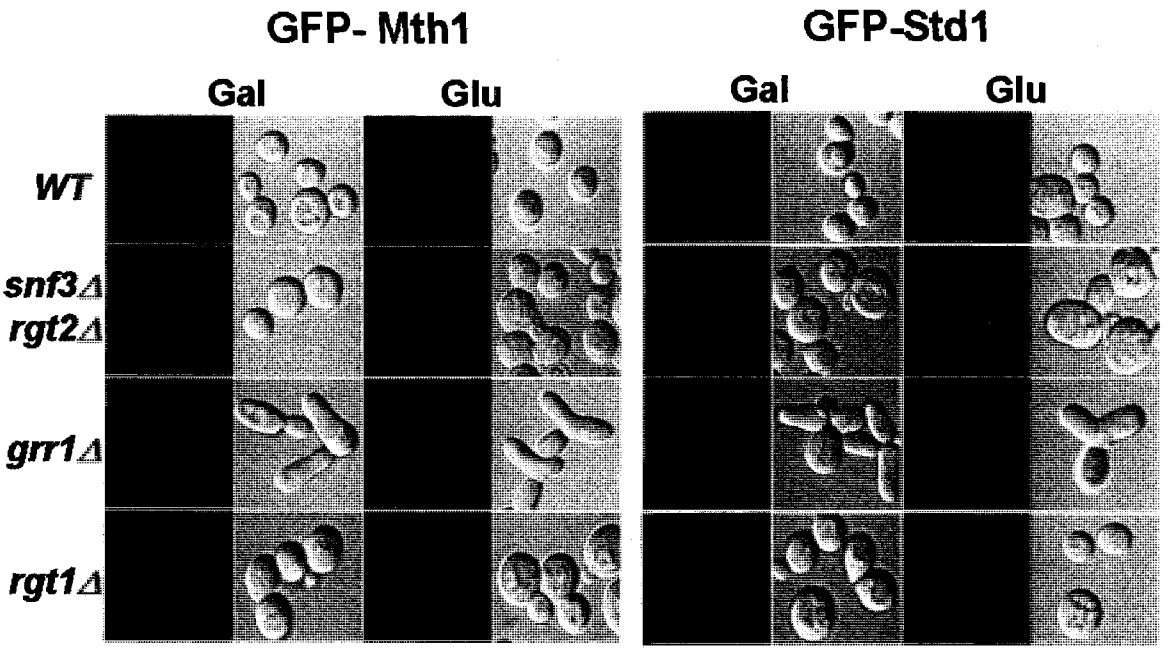


Figure 13. Mth1 and Std1 are present in the nucleus when glucose-induced degradation is prevented by mutational blocks of the Rgt2/Snf3 glucose signaling pathway. Yeast cells of the indicated genotype expressing GFP-Mth1 and GFP-Std1 expressed from the *MET25* promoter on a CEN-based plasmid were grown to mid-log phase in a selective medium containing 2% galactose. Aliquots were then transferred to 2% galactose medium (Gal) or 4% Glucose medium (Glu) and incubated for 60 min. Cells were

(*rgt2Δsnf3Δ and grr1Δ*) and also mutant Mth1 protein (S133A and S133A) is present in nucleus even in glucose grown cells. These results suggest that Mth1 is localized to nucleus and degraded when glucose levels are elevated.

Glucose Does Not Promote Exclusion of Mth1 and Std1 from the Nucleus

To get more convincing evidence that Mth1 degradation may occur in the nucleus, we manipulated the nuclear export and import of Mth1 by tagging the nuclear export signal (NES) of yeast PKIα (Feng *et al.* 1999) and nuclear localization signal (NLS) of SV40 large T antigen (Stochaj *et al.* 1991), respectively and determined cellular levels (Figure 14A and 15A) and subcellular localization (Figure 14B and 15B). GFP-NES (m)-Mth1 (Figure 14A) and GFP-NLS (m)-Mth1 (Figure 15A) behaved like wild-type Mth1 without the tags, suggesting that NES and NLS peptides are functional. Interestingly, NES-Mth1 was degraded in the cytoplasm of the wild-type cells grown in both the presence and absence of glucose (Figure 14A) and it occurred even in the *rgt2Δsnf3Δ* (Figure 14A). In contrast, NLS-Mth1 was degraded by glucose in the glucose sensors-dependent manner (Figure 15A). These results suggest that, although not directly demonstrated, cytoplasmic degradation of Mth1 (presumably also Std1) may occur not by the Rgt2/Snf3 pathway but another degradation mechanism such as vacuolar degradation.

Artificial Exclusion of Grr1 from the Nucleus Prevents Degradation of Mth1

Proteasomal degradation of Mth1 and Std1 appears to require prior ubiquitination

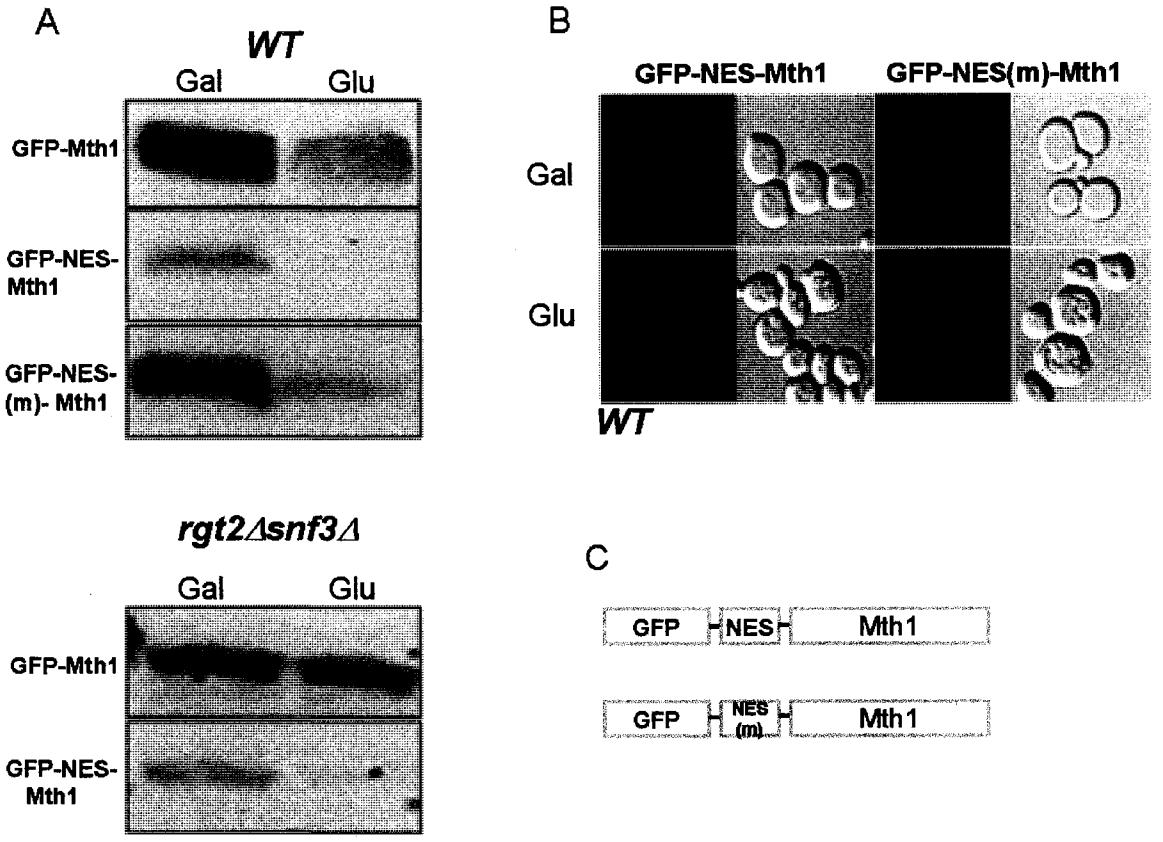


Figure 14. When artificially excluded from the nucleus, Mth1 is degraded in the cytoplasm regardless of the Rgt2/Snf3 pathway. The nuclear export sequence (NES), and the mutant peptide of NES, NES (m) were fused to the N-terminus of Mth1 to make GFP-NES/NES(m)-Mth1, and the resultant proteins were expressed in yeast cells of the indicated genotype. Yeast cells grown in different carbon sources were prepared as described in Figure 13. Cellular levels and subcellular localization of tagged GFP-Mth1 proteins were analyzed by Western blotting using anti-GFP (A) and fluorescent microscopy using CLSM (B), respectively. (C) Construction of GFP-NES/NES (m)-Mth1 is shown.

of the proteins by SCF^{Grr1} (Kim *et al.* 2006; Spielwoy *et al.* 2004; Pasula *et al.* 2007). Grr1 is found in both the nucleus and the cytoplasm; however, deletion of the first 310 amino acids (Grr1-ΔN) that contains a functional NLS localizes the protein to the cytoplasm (Blondel *et al.* 2005). Expression of the truncated Grr1 (Grr1-ΔN; Δ1-280) in the *grr1Δ* mutant restores the morphological defect of the mutant (Li and Johnston 1997)

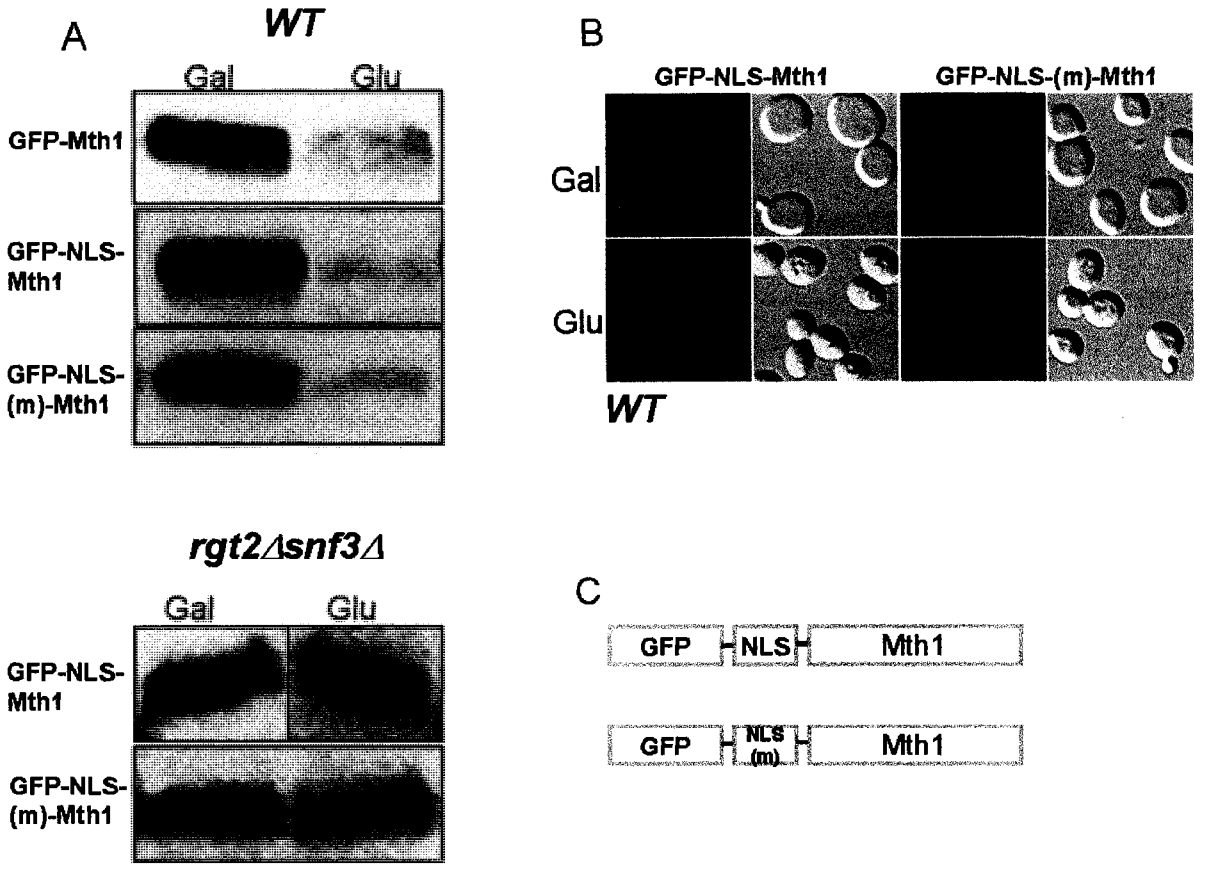


Figure 15. When artificially pushed into the nucleus, Mth1 behaves like wild type Mth1 protein. The nuclear localization sequence (NLS), and the mutant peptide of NLS, NLS (m) were fused to the N-terminus of Mth1 to make GFP-NLS/NLS(m)-Mth1, and the resultant proteins were expressed in yeast cells of the indicated genotype. Yeast cells grown in different carbon sources were prepared as described in Figure 13. Cellular levels and subcellular localization of tagged GFP-Mth1 proteins were analyzed by Western blotting using anti-GFP (A) and fluorescent microscopy using CLSM (B), respectively. (C) Construction of GFP-NLS/NLS (m)-Mth1 is shown.

and has the ability to cause degradation of Gic2 (Blondel *et al.* 2005). We determined whether Mth1 and Std1 are not degraded when Grr1-ΔN (1-280), not the full-length Grr1, is expressed. We could confirm the previous observation (Blondel *et al.* 2005) that GFP-Grr1-ΔN (Δ1-280) was localized to cytoplasm (Figure 16A) and the expression of this truncated protein restored the morphological defect of the *grr1Δ* mutant (Figure 16A).

However, degradation of GFP-Mth1 and GFP-Std1 by the glucose was not observed (Figure 16B) and the proteins were found in the nucleus (Figure 16C) in the *grr1Δ* mutant expressing Grr1-ΔN (1-280). Also *HXT1* expression was not repressed in *grr1Δ* mutant expressing Grr1-ΔN (Figure 18), which was expected because Mth1 and Std1 are not degraded. *HXT1* expression in these cells could be because Mth1 and Std1 may not be interacting with Rgt1 and thus relieving Rgt1 from the promoters of *HXT* genes. These results suggest that Mth1 and Std1 are not excluded from the nucleus in response to glucose.

Taken together, Mth1 and Std1 did not seem to shuttle dynamically between the nucleus and the cytoplasm as suggested previously (Johnston and Kim 2005; Moriya and Johnston 2004); rather they are likely to be present in the nucleus when glucose is absent and degraded in the nucleus when glucose levels are increased. Also presence of nuclear Grr1 is required for the nuclear degraded of Mth1 and Std1. These observations led us to reconsider our previous hypothesis on phosphorylation of Mth1 and Std1 by the membrane-tethered Yck1/2.

Degradation of NLS-Mth1 in the Nucleus Requires Grr1

Mth1 and Std1 are degraded in response to glucose and this degradation is mediated by SCF^{Grr1}-26S proteasome and the above result suggests that presence of Grr1 in the nucleus is required for the nuclear degradation of these proteins (Figure 16). To provide more evidence, I expressed GFP-NLS-Mth1 (which localizes the protein to the nucleus) in wild-type and *grr1Δ* cells and measured cellular levels of the protein using Western blotting. NLS-GFP-Mth1 was degraded in the nucleus in wild-type cells but was

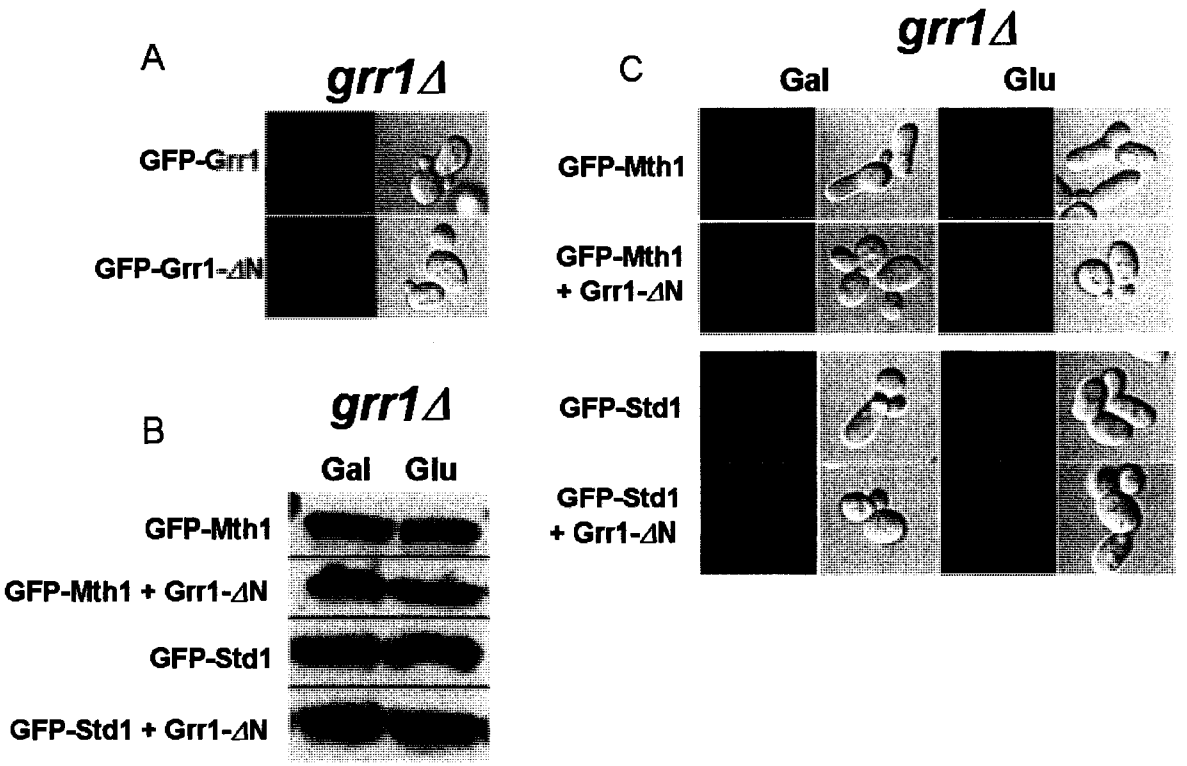


Figure 16. Glucose induced degradation of Mth1 is prevented when Grr1 is artificially excluded from the nucleus. (A) Grr1 and Grr1-Δ-N (lacking the first 280 amino acids of Grr1) were fused to GFP and the wild-type cells (FM391) expressing the resultant proteins were observed by fluorescent microscopy. (B) Cellular levels of GFP-Mth1 and GFP-Std1 in cells expressing Grr1 and Grr1-Δ-N were measured by Western blotting using anti-GFP antibody. (C) GFP-Mth1 and GFP-Std1 were coexpressed with Grr1-Δ-N in *grr1Δ* and subcellular localization of GFP-Mth1 and GFP-Std1 was observed by fluorescent microscopy.

resistant to degradation in *grr1Δ* cells (Figure 17). *RGT2-1* causes glucose independent degradation of Mth1 and Std1 (Chapter IV). Expression of GFP-NLS-Mth1 in *RGT2-1* also caused degradation of nuclear Mth1 (GFP-NLS-Mth1) independent of glucose (data not shown). These data clearly demonstrate that Mth1 (and perhaps Std1) are degraded in the nucleus and presence of Grr1 in the nucleus is required for this degradation.

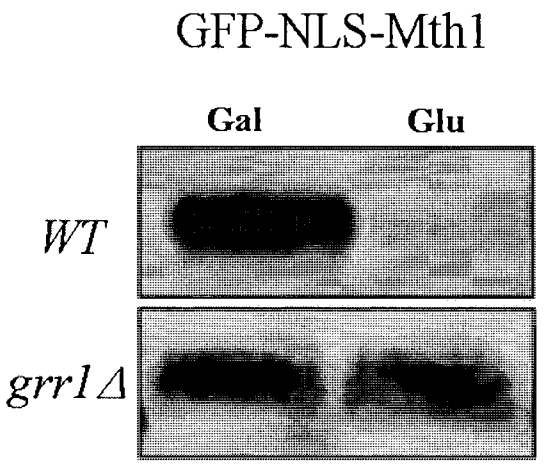


Figure 17. Degradation of nuclear Mth1 requires Grr1. Mth1 tagged with nuclear localization signal (NLS and GFP) at the N-terminus was expressed in yeast cells of the indicated genotype. Yeast cells expressing GFP-NLS-Mth1 plasmid were grown to mid-log phase in selective medium containing 2% galactose. Aliquots were then transferred to 2% galactose medium (Gal) or 4% glucose medium (Glu) and incubated for 60 min. Cellular levels of GFP-NLS-Mth1 proteins were determined using anti-GFP antibody.

Membrane Tethering of Yck1/2 Does Not Have a Significant Role in the Degradation of Mth1

Yck1 and Yck2 are shown to be associated with the cell membrane through palmitoylation by Akr1 (Feng and Davis 2000; Babu *et al.* 2004). Carboxy-terminal tagging of proteins modified with palmitoyl and farnesyl groups appears to disturb their function (Sun *et al.* 2004; Roth *et al.* 2002). Yck1/2 are known to be tethered to the plasma membrane through palmitoylation of C-termianl Cys-Cys sequence by the palmitoyl transferase Akr1 (Sun *et al.* 2004; Roth *et al.* 2002). Therefore, we tagged GFP to the amino-terminus of Yck1/2. GFP-Yck1 and GFP-Yck2 were found to be associated with the plasma membrane in wild-type cells, but they localized diffusely through the entire cell when *AKR1* was disrupted (Figure 19A) as reported previously (Sun *et al.*

2004; Roth *et al.* 2002). Since coupling of Yck1/2 to the glucose sensors has been postulated as an essential regulatory step in the Rgt2/Snf3 pathway, we determined whether *AKR1* deletion prevents degradation of Mth1 and Std1. Unexpectedly, glucose normally promoted degradation of Mth1 and Std1 (Figure 19B) and indeed induced expression of the *HXT1* gene in the *akr1Δ* strain (Figure 19C).

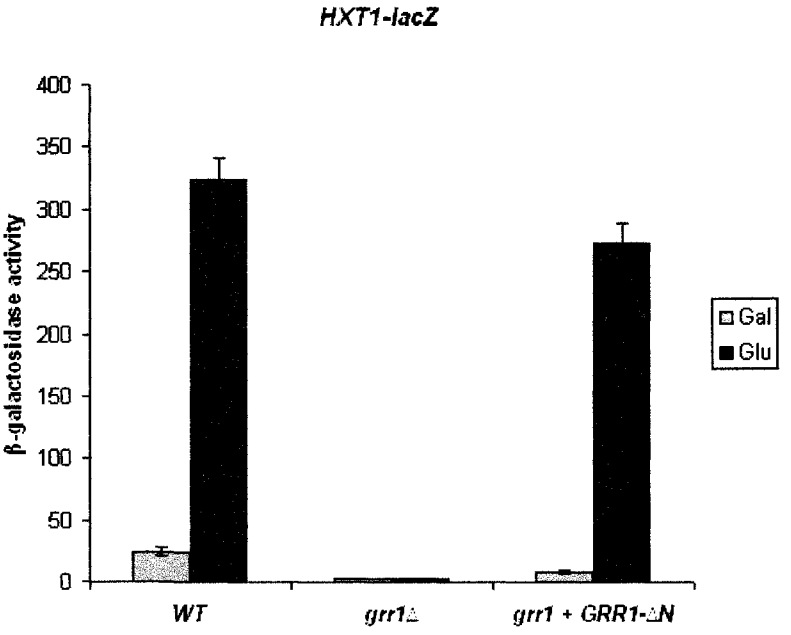
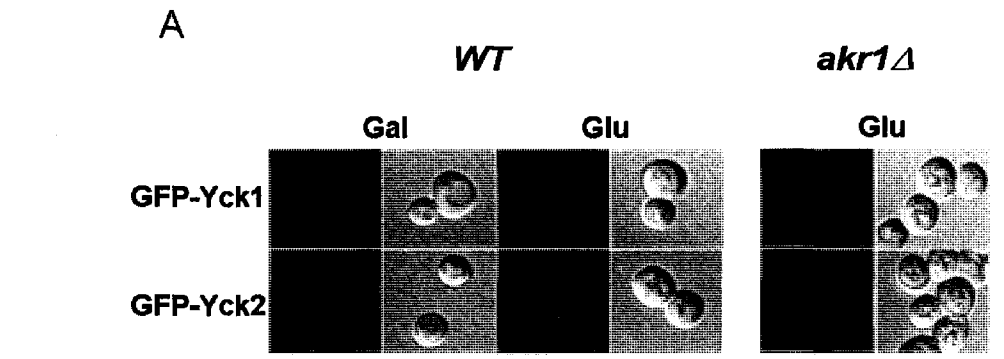


Figure 18. Prevention of degradation of Mth1 and Std1 by cytoplasmic Grr1 (Grr1-Δ-N) does not repress *HXT1* expression. Yeast cells of indicated genotype (*WT* and *grr1Δ*) expressing *HXT1-lacZ* and *HXT1-lacZ* and Grr1-Δ-N (*grr1Δ* + GRR1-ΔN) were grown in 2% galactose medium and at mid-log phase aliquots were transferred to 2% galactose and 4% glucose and incubated for 1hr and assay was performed as described previously (Kaniak *et al.* 2004). Results are reported in Miller Units (materials and methods). The reported enzyme activities were averages of the results from triplicate of three different transformants.



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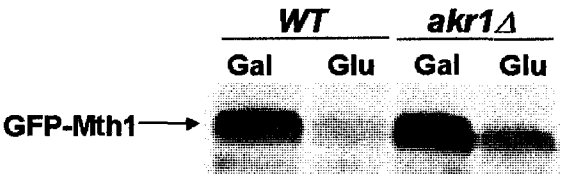


C

HXT1-lacZ

Genotype	Gal	Glu
WT	1.2	638.3
akr1Δ	2.0	63.9
yck1Δyck2-ts	1.1	2.1

β-galactosidase (Miller Unit)

Figure 19. Coupling of Yck1 and Yck2 to the glucose sensors may not be crucial for the Rgt2/Snf3- glucose signaling pathway. (A) Subcellular localization of GFP-Yck1 and GFP- Yck2 in wild type and *akr1Δ* strains were observed by fluorescent microscopy. GFP-Yck1 and GFP-Yck2 were expressed from the *MET25* promoter on a CEN-based plasmid (Kim *et al.* 2006). (B) Glucose normally induces degradation of Mth1 and Std1 in *akr1Δ* strains. Cellular levels of GFP-Mth1 were analyzed by Western blotting using anti-GFP antibody. (C) *AKR1* deletion does not affect glucose induction of *HXT1* expression. Expression of the *HXT1* gene in the absence of (Gal) and the presence of glucose (Glu) was measured by assaying β-galactosidase activity expressed from the *HXT1*-lacz reporter.

Yck1 and Yck2 Activity is Not Carbon Source Dependent

Yck1 and Yck2 are thought to phosphorylate Mth1 and Std1 in presence of glucose (Moriya and Johnston 2004). So, we tested if Yck1 and Yck2 are differently regulated in presence and absence of glucose. First we checked the cellular levels of Yck1 and Yck2 in presence and absence of glucose. Western blot analysis indicated that protein levels are not regulated by glucose or by glucose sensors (Figure 20A). To know

whether glucose regulates the activity of the yeast CK1, Yck1-His-ProA was harvested from the glucose- or galactose- grown cells and tested for its ability to phosphorylate Gst-Mth1 expressed in *E. coli.* Our autoradiography indicated that they were almost equally active in phosphorylating Gst-Mth1 *in vitro* (Figure 20B).

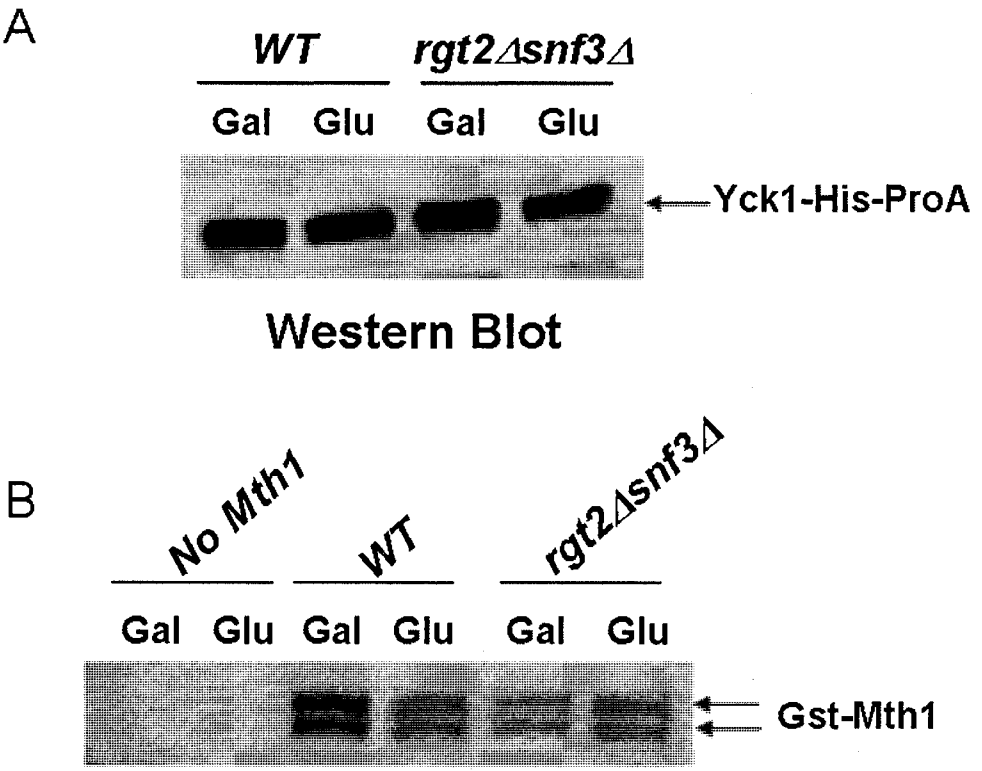


Figure 20. Yeast Casein Kinases Yck1 and Yck2 are constitutively active (A) Cellular levels of Yck1 and Yck2 are not significantly changed by glucose or deletion of the glucose sensor genes (*rgt2Δsnf3Δ*). Yck1-His-ProA expressed from wild type and *rgt2Δsnf3Δ* strains were detected by Western blotting using the anti-ProA antibody. (B) Yck1 and Yck2 are active regardless the presence of glucose *in vitro*. For the kinase assay of Yck1 *in vitro*, Yck1-His-ProA was immunoprecipitated using anti-ProA from the yeast cell extracts and Gst-Mth1 was affinity purified using glutathione-Sepharose-4B beads from *E. coli* cell extracts, respectively. After incubating with [γ^{32}P]ATP, Yck1-His-ProA and Gst-Mth1 were resolved in SDS-PAGE gels and detected by autoradiography. Usually two radiolabelled protein bands corresponding to Gst-Mth1 were detected; the lower bands are thought to be the degradation products of the full length Gst-Mth1.

Collectively, these results suggest that targeting of Yck1/2 to the cell membrane and indeed coupling of Yck1/2 to the glucose sensors are not necessary for the degradation of Mth1 and Std1. We also determined whether there are minimal levels of Yck1/2 in the nucleus by fractionation of cellular components (Kipper *et al.* 2002); however, the nuclear fraction did not contain the full-length GFP-Yck1 and GFP-Yck2 (data not shown)

The results in this chapter suggest that Mth1 (and perhaps Std1) are degraded in nucleus in response to glucose and present in the nucleus when there is no glucose. Presence of nuclear Grr1 is necessary for the nuclear degradation of Mth1 and Std1. Interestingly, we also found that plasma membrane tethering of Yck1 and Yck2 is not required for the glucose induced *HXT1* expression and nuclear degradation of Mth1. It is not yet clearly known how the glucose sensors activate Yck1 and Yck2 kinases. Further work is needed to determine how Rgt2 and Snf3 transmit signal to Yck1 and Yck2.

CHAPTER VI

TRANSCRIPTOMIC ANALYSIS OF THE *mth1Δstd1Δ* MUTANT

In presence of extracellular glucose, two plasma membrane receptor proteins Snf3 and Rgt2 generate intracellular signal which ultimately leads to the induction of *HXT* genes expression. This is achieved by relieving the transcription factor, Rgt1from the promoters of *HXT* genes by Rgt2/Snf3-Rgt1 signaling pathway (Ozcan and Johnston 1999; Forsberg and Ljungdahl 2001). In the absence of glucose, the Rgt1 DNA-binding repressor represses *HXT* expression in conjunction with Mth1 and Std1 that physically interact with Rgt1 (Tomas-Cobos and Sanz 2002; Lakshmanan *et al.* 2003; Polish *et al.* 2003). Glucose disrupts this interaction by promoting degradation of Mth1 and Std1 (Flick *et al.* 2003; Moriya and Johnston 2004; Kim *et al.* 2006), thereby relieving repression of *HXT* expression (Flick *et al.* 2003; Mosley *et al.* 2003; Kim *et al.* 2003). So indirectly Mth1 and Std1 serve as transcriptional regulators of *HXT* genes.

To identify other (in addition to the known hexose transporter genes) target genes whose expression is regulated by Mth1 and Std1, I did microarray analysis with *mth1Δstd1Δ* mutant grown in YEP + 2 % galactose medium. I identified different functional categories of genes which show differential expression in *mth1Δstd1Δ* mutant. Microarray analysis also identified previously known target genes (induction in the expression of *HXTs*) of Mth1 and Std1, which serve as reference for this microarray analysis. An analysis of the promoters of the genes shows that, 63 of 89 identified genes show a strong match to the consensus Rgt1 binding site $_{5'}$CGGANNA$_{3'}$ (Kim *et al.* 2003) and each of these had one or more of the Rgt1 consensus sites. It was also surprising that the microarray analysis identified genes with no Rgt1 binding sites in their promoters.

This suggests that Mth1 and Std1 may be functioning through other yet unidentified transcriptional factors or may be themselves serving as transcriptional regulators for these genes.

The Functional Categories of Genes which Show Differential Expression in *mth1Δstd1Δ* Mutant

1. Mitochondrial/respirational genes (21)
2. Transporter and membrane proteins encoding genes (17)
3. Amino acid pathway genes (9)
4. Ribosomal genes (5)
5. Transcriptional factors (4)
6. Kinases (2)
7. Other functions /unknown function genes (31)

Table 3. Functional categories of genes identified by microarray analysis of *mth1Δstd1Δ* mutant grown in YEP + 2% galactose.

Functional Category	GENE/ORF Name
Mitochondrial/respiration	MDH1 MAM33 MDH2 ACO1 IDH1 POR1 IDH2 YMC2 YAL046C MSK1 YMC2 QCR2 OAC1 ISF1 MIC17 YCP4 IDP1 KGD2 GND2 SOD2 TAL1
Amino acid pathway	ASP1 URA1 URA2 SAH1 HIS4 LEU1 MET14 STR3 ECM17
Transport and Membrane proteins	FET3 HXT1 HXT2 HXT3 HXT4 HXT6 HXT7 MCH5 ZRT2 OAC1 FET4 YMC2 FET3 IZH4 STE2 ASG7 GSP1
Ribosomal	RPS0B RPL6B RPS15 RPL5 RPL43A
Kinases	MRK1 MOH1
Transcription	TEC1 MIG3 MIG2 RPB8
Genes with other functions and unidentified functions	OPI11 YPR063C NOC4/UTP19 CWP1 YCL027C PM140 LSB1 YPL066W HEM2 ARC1 PHM8 VEL1 SNZ1 YHR033W BAR1 GVP1 RNR2 STM1 TFS1 GSP1 ERG13 HSC82 YGP1 SSB2 YNL234W YKR075C YLR108C RIB4 GSP2 YOR387C YOR062C

To validate microarray analysis results, I determined the expression levels of major functional categories of genes identified (mitochondrial/respirational genes, Amino acid pathway genes and few transporter genes) by real time pcr (RT-PCR) quantification analysis and *ACT1* (Brickner *et al.* 2007) served as endogenous control. For RT-PCR analysis, I grew wild-type and *mth1Δstd1Δ* (KY33) cells in YEP + 2% galactose and harvested the cells in early logarithmic phase for RNA isolation. For RT-PCR, I used *HXT1* as a control which is one of the previously known target gene of Rgt2/Snf3-Rgt1 pathway and is induced several fold in cells deleted for *MTH1* and *STD1*. As expected, I observed approximately 22 fold induction of *HXT1* expression in *mth1Δstd1Δ* mutant when compared to wild-type cells (data not shown). RT-PCR for transport genes is shown in figure 21. Expression pattern for all these genes tested agreed with the microarray analysis results. Significant expression differences were observed for *MCH5*

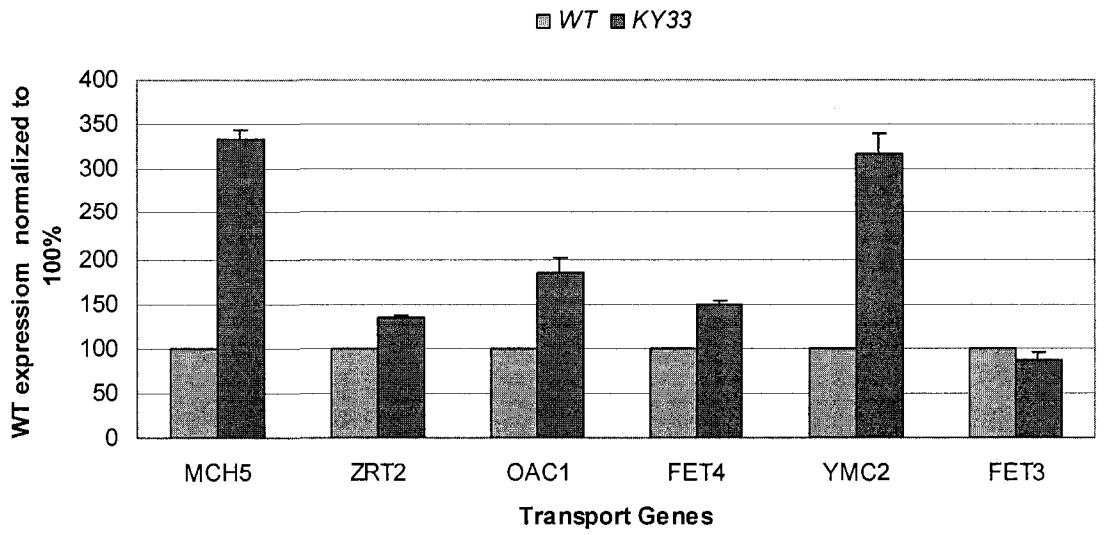


Figure 21. Transcriptomic analysis of *mth1Δstd1Δ* identified new transporter target genes. RT-PCR of indicated transporter genes in wild-type and *mth1Δstd1Δ* (KY33) cells. The cells were grown in YEP + 2% galactose to early logarithmic phase and harvested for RNA isolation. RNA isolation and RT-PCR reaction was done as described in materials and methods. Wild-type expression was set to 100% and values are mean from two independent experiments.

and *YMC2* (more than 3 fold induction of expression in the *mth1Δstd1Δ* mutant cells) and to little less extent for *OAC1* genes (~ 2 fold induction).

MCH5 encodes for plasma membrane riboflavin transporter and facilitates the uptake of vitamin B2. Mch5 is required for FAD-dependent processes (*Saccharomyces* Genome Database, SGD). It shows sequence similarity to mammalian monocarboxylate permeases (Reihl and Stolz 2005). Promoter analysis of *MCH5* shows the match for 3 consensus Rgt1 binding sites. *YMC2* encodes a mitochondrial protein and it is a putative inner membrane transporter with a role in oleate metabolism and glutamate biosynthesis (el Moualij *et al.* 1997; Trotter *et al.* 2005). *OAC1* encodes for mitochondrial inner membrane transporter, transports oxaloacetate, sulfate, thiosulfate, and isopropylmalate and it is a member of the mitochondrial carrier family (Palmieri *et al.* 1999; Marobbio *et al.* 2008). *OAC1* promoter has 3 Rgt1 binding consensus sites. Fet3 and Fet4 are high affinity and low affinity plasma membrane iron transporters respectively (SGD). Zrt2 is a low affinity plasma membrane zinc transporter (SGD). Mth1 and Std1 in addition to regulating hexose transporter genes appear to also regulate the expression of other transport genes. Further characterization is necessary to understand the exact role of Mth1 and Std1 in controlling the transcript levels of these transporter genes.

RT-PCR validation for the identified amino-acid genes is shown in figure 22. Significant expression change is observed for *URA1* gene (a decrease of 2 fold expression in *mth1Δstd1Δ* (KY33)) cells compared to wild-type cells. *URA1* encodes dihydroorotic acid dehydrogenase an enzyme involved in the *de novo* synthesis of pyrimidine ribonucleotides (Vorisek *et al.* 2002; Roy 1992). *URA1* promoter has 1 Rgt1 binding consensus site.

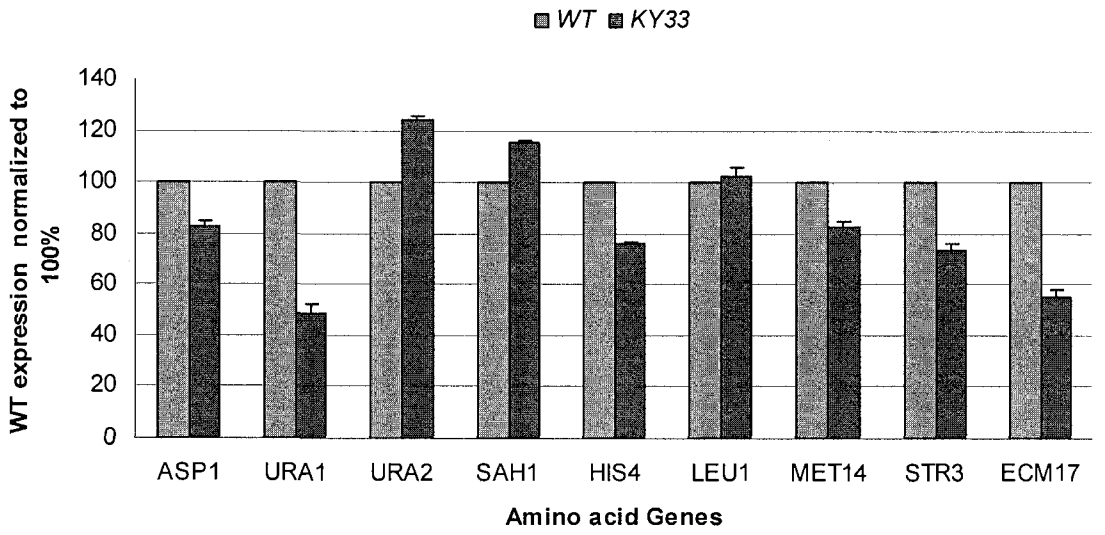


Figure 22. Transcriptomic analysis of *mth1Δstd1Δ* identified new amino acid pathway target genes. RT-PCR of indicated genes in wild-type and *mth1Δstd1Δ* cells. The cells were grown in YEP + 2% galactose to early logarithmic phase and harvested for RNA isolation. RNA isolation and RT-PCR reaction was done as described in materials and methods. Wild-type expression was set to 100% and values are mean from two independent experiments.

Microarray analysis of *mth1Δstd1Δ* also identified mitochondrial/respiration genes. RT-PCR validation results of tested mitochondrial genes are shown in figure 23. RT-PCR results of most of the genes identified agree with microarray results except *SOD2*, *YCP4* and *ACO1*. These three genes show an increase in the levels of expression in *mth1Δstd1Δ* mutant. *SOD2* encodes a manganese-superoxide dismutase (MnSOD) that is localized to the mitochondrial matrix and is involved in oxygen radical detoxification (SGD). *SOD2* expression is positively regulated by the heme-dependent activator Hap 2-3-4-5 complex and the heme binding transcription activator, Hap1p (Flattery-O'Brien *et al.* 1997; Pinkham *et al.* 1997). *SOD2* promoter has two Rgt1-binding consensus sites. *YCP4* encodes protein of unknown function which has sequence and structural similarity to flavodoxins (SGD). The protein was detected in highly purified mitochondria in high-

throughput studies (Reinders *et al.* 2006). *ACO1* encodes for aconitase which is required for the tricarboxylic acid (TCA) cycle and also independently required for mitochondrial genome maintenance (Gangloff *et al.* 1990; Chen *et al.* 2005). *ACO1* expression is increased by approximately three fold in *mth1Δstd1Δ* mutant cells. Promoter analysis reveals the presence of 3 Rgt1-binding consensus sites.

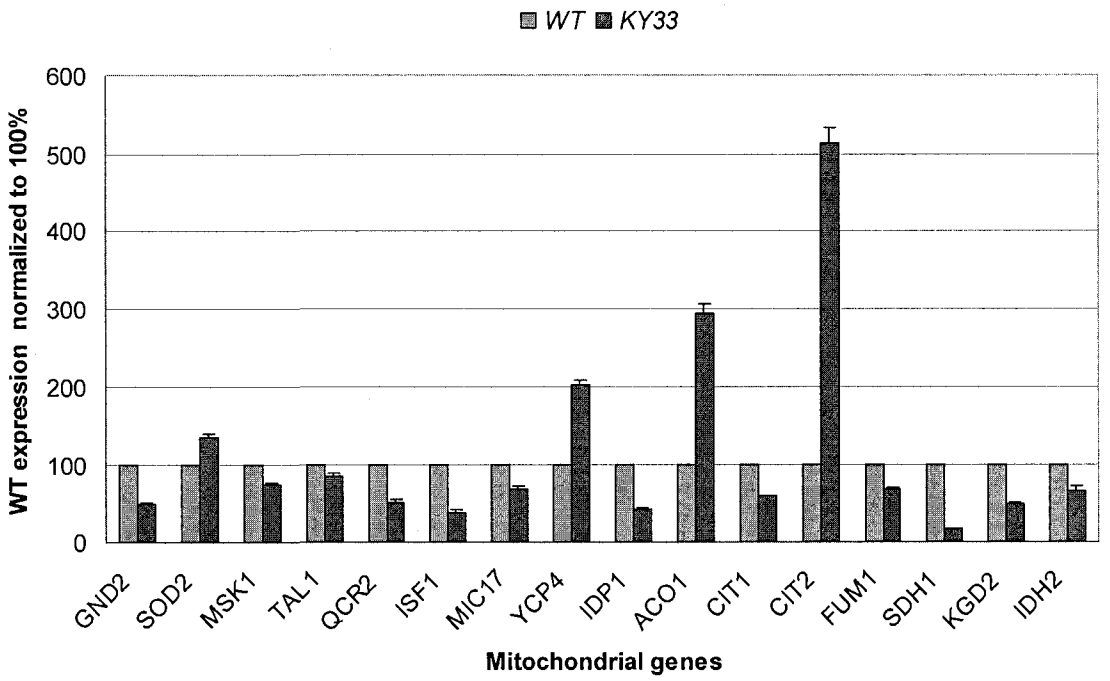


Figure 23. Transcriptomic analysis of *mth1ΔStd1Δ* identified new mitochondrial-respiration target genes. RT-PCR of indicated genes in wild-type and *mth1Δstd1Δ* (KY33) cells. The cells were grown in YEP + 2% galactose to early logarithmic phase and harvested for RNA isolation. RNA isolation and RT-PCR reaction was done as described in materials and methods. Wild-type expression was set to 100% and values are mean from two independent experiments.

The other mitochondrial genes tested by RT-PCR except *CIT2* showed a decrease in the expression level in *mth1Δstd1Δ* mutant. Mitochondrial genes which showed significant differential expression (2 fold or more) include *GND2*, *QCR2*, *ISF1*, *IDP1*,

S*DH1* and *KGD2*. Mth1 and Std1 separately or together appear to positively regulate the expression of these genes.

QCR2 encodes subunit 2 of the ubiquinol cytochrome-c reductase complex, which is a component of the mitochondrial inner membrane electron transport chain and its transcription is regulated by Hap1p, Hap2p/Hap3p, and heme (Dorsman and Grivell 1990). *QCR2* is conserved across eukaryotes (Trumpower 1990) and is homologous to human UQCRC2. *ISF1* encodes for a Serine-rich, hydrophilic protein with similarity to Mbr1p. Overexpression of Isf1 suppresses growth defects of hap2, hap3, and hap4 mutants. *ISF1* expression is decreased more than two fold in *mth1Δstd1Δ* mutant; however its expression drastically (16 fold) decreased when expressed in glycerol medium (data not shown). *ISF1* expression is not dependent on Std1 function but it is dependent on Mth1 (Figure 24 and 25). RT-PCR of *ISF1* in *std1Δ* and *mth1Δ* separately grown in galactose medium shows a reduced level of expression only in *mth1Δ* cells. *IDP1* encodes for mitochondrial NADP-specific isocitrate dehydrogenase and it catalyzes the oxidation of isocitrate to alpha-ketoglutarate. Its expression is reduced more than two fold in the mutant cells.

SDH1 encodes for flavoprotein subunit of succinate dehydrogenase 1. Sdh1 couples the oxidation of succinate to the transfer of electrons to ubiquinone (Oyedotun and Lemire 2004). There is significant decrease (~6 fold) in the expression of *SDH1* in *mth1Δstd1Δ* mutant cells (Figure 23). Individual deletion (*std1Δ* and *mth1Δ*) cells also cause 2 fold decrease (Figure 24 and 25) in the expression of *SDH1*, but abolishing the function of both Mth1 and Std1, causes more drastic expression change (6 fold). *KGD2*

encodes for dihydrolipoyl transsuccinylase protein. It is a component of the mitochondrial alpha-ketoglutarate dehydrogenase complex, which catalyzes the oxidative

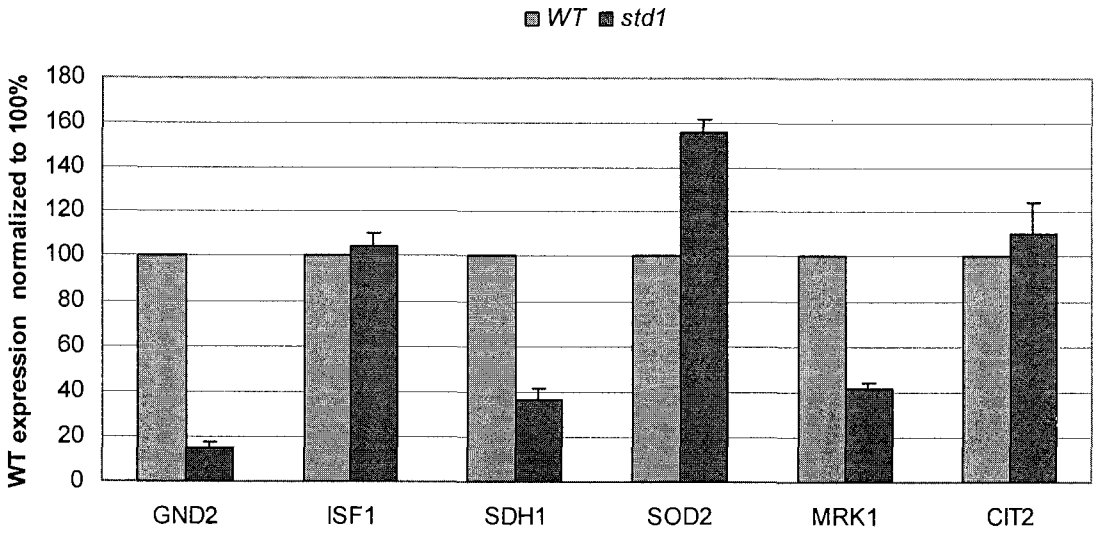


Figure 24. RT-PCR of indicated genes in wild-type and *std1Δ* cells. The cells were grown in YEP + 2% galactose to early logarithmic phase and harvested for RNA isolation. RNA isolation and RT-PCR reaction was done as described in materials and methods. Wild-type expression was set to 100% and values are mean from two independent experiments.

decarboxylation of alpha-ketoglutarate to succinyl-CoA in the TCA cycle (Repetto and Tzagoloff 1990).

CIT2 encodes for Citrate synthase enzyme (Kim *et al.* 1986). Cit2 catalyzes the condensation of acetyl coenzyme A and oxaloacetate to form citrate. *CIT2* expression is controlled by Rtg1p and Rtg2p transcription factors (Liao and Butow 1993). *CIT2* expression increases over 5 fold in galactose grown cells lacking *MTH1* and *STD1* (Figure 23). Its expression increased 2 fold in cells lacking *MTH1* but not in cells lacking *STD1* (Figure 25 and 24). Promoter analysis of *CIT2* gene showed the presence of 4 Rgt1-binding consensus sites. Std1 interacts with the transcription factor, Rtg2 (SGD and

BioGRID protein data base, Toronto) which may be responsible for the differential expression of *CIT2* in *mth1Δstd1Δ* mutant cells.

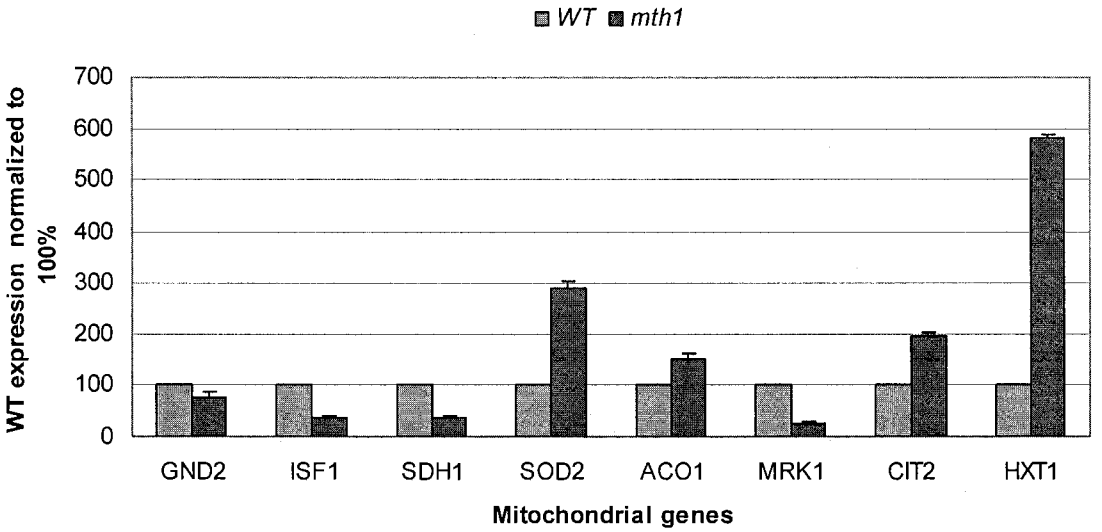


Figure 25. RT-PCR of indicated genes in wild-type and *mth1Δ* cells. The cells were grown in YEP + 2% galactose to early logarithmic phase and harvested for RNA isolation. RNA isolation and RT-PCR reaction was done as described in materials and methods. Wild-type expression was set to 100% and values are mean from two independent experiments.

In addition to the above genes discussed, the expression of *MRK1* also changes significantly. There is 3 fold decrease in expression in *mth1Δstd1Δ* mutant cells. *MRK1* encodes for glycogen synthase kinase 3 and functions to activate Msn2p-dependent transcription of stress responsive genes and that function in protein degradation (Hardy *et al.* 1995; Andoh *et al.* 2000).

Std1 shows interaction with Hap2 and Hap5 which are part of CBF, a transcriptional activation complex. CBF stands for CCAAT-binding factor and is a multimeric complex, composed of Hap2/Hap3/Hap4 and Hap5 proteins. CBF is one of the transcriptional activators responsible for the activation of the genes involved in respiratory metabolism (DeRisi *et al.* 1997; Gancedo 1998 and Schuller 2003). Std1

interaction with the proteins of the CBF complex may be directly or indirectly playing a role in the transcriptional activation of respiratory genes (like *SAH1*, *KGD2,SOD2* and others) identified by the microarray analysis of *mth1Δstd1Δ*.

Transcriptomic analysis of *mth1Δstd1Δ* identified new functional categories of target genes for transcriptional repressor proteins Mth1 and Std1. But further characterization is needed to establish the details of specific roles of these proteins in regulating the newly identified target genes.

CHAPTER VII

DISCUSSION

In the budding yeast, *Saccharomyces cerevisiae* glucose induces the expression of *HXT* genes which encode for hexose transporters and facilitate the glucose uptake. This is achieved via Rgt2/Snf3-Rgt1 glucose signaling pathway. Rgt2 and Snf3 are glucose sensors involved in generating an intracellular glucose signal. *RGT2* is required for the maximal expression of the high glucose-induced *HXT1* gene, and *SNF3* is required for the low glucose-induced expression of *HXT2* and *HXT4* (Ozcan *et al.* 1996). Extracellular glucose causes conformational change in glucose sensor proteins which in turn generate signal transduction leading to the induction of *HXT* expression. This idea is strengthened by the observation that dominant mutations in *RGT2* (*RGT2-1*: Arg231 ⟶ Lys) and SNF3 (*SNF3-1*: Arg229 ⟶ Lys) cause constitutive expressions of several glucose induced *HXT* genes (Ozcan *et al.* 1996). It is thought that this mutation causes Rgt2 and Snf3 always to be in "signaling competent" conformation, thus causing constitutive production of an intracellular glucose signal that activates *HXT* gene expression (Ozcan *et al.* 1996). However, the mechanism by which *HXTs* are expressed in *RGT2-1* and *SNF3-1* has not been shown biochemically.

In chapter IV, I show that glucose-independent expression of *HXT* in *RGT2-1* and *SNF3-1* is achieved by the degradation of paralogous proteins Mth1 and Std1 (Figure 5). This glucose-independent degradation is achieved by similar mechanism in which Mth1 and Std1 are degraded in presence of glucose. *RGT2-1* and *SNF3-1* promoted degradation requires phosphorylation of Mth1 and Std1 by yeast casein kinases, Yck1 and Yck2 (Figure 9), Ubiquitination by SCF^{Grr1}-ligase complex (Figure 7). The data supports the

view that *RGT2-1* locks the protein in the glucose-bound conformation, and thus causing constitutive activation of the glucose sensor signaling pathway (Ozcan et al. 1996). Verification of glucose-independent degradation of Mth1 and Std1 is essential to avoid defining incorrect models. For instance, we cannot rule out the possibilities that: 1) *RGT2-1* and *SNF3-1* simply inactivate Rgt1 (there is ample evidence that Mth1 and Std1 work together with Rgt1, the *HXT* gene repressor); and 2) *RGT2-1* and *SNF3-1* promote nuclear export of Mth1 and Std1. In these two models, expression of *HXT* genes is still induced without the degradation of Mth1 and Std1. My data rules out the above two models and provides biochemical evidence that *RGT2-1* and *SNF3-1* induce degradation of Mth1 and Std1 even in the absence of glucose.

In the second part of my dissertation, I tested the proposed model of the Rgt2/Snf3 glucose signaling pathway by determining the role of membrane tethered Yck1/2 in the pathway and also determining the subcellular localization of Mth1 and Std1. Taken together my data suggests that membrane tethering of Yck1/2 is not absolutely necessary for the glucose-induced degradation of Mth1. While arguing against a role for the kinases in the event, my results could provide alternative explanations to the previously reported results that: 1) interaction between the glucose sensors and Yck1/2 is constitutive, not glucose regulated; 2) the tails of the glucose, which have been thought to recruit Mth1 and Std1 to the vicinity of Yck1/2, are not essential for the glucose signaling, because Rgt2 without the tail can generate the glucose signal if it is overexpressed (Moriya and Johnston 2004).

I confirmed the insignificant role of membrane tethered Yck1/2 in the degradation of Mth1 (and perhaps Std1) in *akr1Δ* strain, in which Yck1/2 fail to localize to the plasma

membrane (Figure 19). These observations are in contrary to the previous study that Yck1/2 are essentially required for the degradation of Mth1 and Std1 (Moriya and Johnston 2004). Most of the targets of Yck1/2 seem likely to be plasma membrane proteins. They are responsible in phosphorylation of the PEST-like ubiquitination-endocytosis signal of the mating pheromone receptors Ste2 (Hicke 1999) and Ste3 (Panek *et al.* 1997; Feng and Davis 2000), and uracil permease Fur4 (Marchal *et al.* 1998). The kinases are also known to regulate activity of the maltose permease (Gadura *et al.* 2006), a multidrug transporter Pdr5 (Egner and Kuchler, 1996), and H+-ATPase (Estrada *et al.* 1996). Therefore, it remains possible that Yck1/2 promote glucose-induced endocytosis of Rgt2 and Snf3 that triggers degradation of Mth1 and Std1.

However, Yck1/2 also appear to be involved in amino acid signaling. Extracellular amino acids induce endoproteolytic processing of Stp1 and Stp2 through the plasma membrane-localized Ssy1- Ptr3-Sssy (SPS) sensor (Andreasson and Ljungdahl 2002). The processing requires Yck1/2 and SCF^{Grr1}, but not the proteasome (Andreasson and Ljungdahl 2002; Abdel-Sater *et al.* 2004).

Our lab data of mutational analysis of the Yck1/2 phosphorylation sites in Mth1 and Std1 shows that not only Mth1 and Std1 are differentially phosphorylated, but also the serine residues within the cluster of Mth1 are not equally phosphorylated. Taken together, these results support an idea that Mth1 and Std1 are likely to be phosphorylated by an unidentified kinase that may be recognizing similar phosphorylation consensus sites as of Yck1/2.

Glucose also regulates Rgt1 function by promoting phosphorylation of Rgt1. Rgt1 has been shown to be phosphorylated and negatively regulated by the PKA (Protein

kinase A) in high glucose; deletion of the *TPK* genes encoding PKA and elimination of the putative PKA phosphorylation sites in the Rgt1 inhibit dissociation of Rgt1 from DNA, and indeed prevents induction of *HXT* expression (Kim and Johnston 2006). Artificial exclusion of Grr1 from the nucleus prevents degradation of Mth1 and Std1 (Figure 16), but induces *HXT1* expression (Figure 18). While not determined, Rgt1 could be phosphorylated by PKA in high glucose and inactivated for its repressor function, such that *HXT1* is expressed in spite of presence of high levels of Mth1 and Std1 in the nucleus (Figure 16). Interestingly, Yck1/2 are known to physically interact with PKA (Ho Y *et al.* 2002), raising a possibility that Yck1/2 might play a role in PKA-Rgt1 pathway.

In presence of glucose sensor proteins Rgt2 and Snf3 generate an intracellular signal which ultimately leads to the expression of induction of *HXT* expression. In absence of glucose, the Rgt1 DNA-binding repressor represses *HXT* expression in conjunction with Mth1 and Std1, paralogous proteins that physically interact with Rgt1 (Tomas-Cobos and Sanz 2002; Lakshmanan *et al.* 2003; Polish *et al.* 2005). Mth1 and Std1 serve as transcriptional regulators of *HXT* genes along with Rgt1. Microarray analysis with *mth1Δstd1Δ* identified different functional categories of genes (Table 3 and Appendix B) in addition to genes encoding for hexose transporters. Major categories of genes include 1) Transporter genes, 2) Amino acid pathway genes and 3) Respiration genes. Validation of microarray results by RT-PCR for transporter genes showed an induction in the expression of *MCH5*, *ZRT2*, *OAC1*, *FET4* and *YMC2* transporter genes in *mth1Δstd1Δ* mutant cells grown in no glucose condition (galactose). This implies a regulatory role of Mth1 and Std1 in the expression of other transporter genes in addition to hexose transporter genes (*HXT*s). The other major functional category of genes

identified by this microarray analysis includes mitochondrial/respirational genes.

Saccharomyces cerevisiae is respirofermentative yeast. It represses respiratory metabolism when growing in glucose as the sole carbon source, even in presence of oxygen (Flores *et al.* 2000). Following glucose depletion, cells undergo a major reprogramming of gene expression, known as diauxic shift, to activate the genes that encode proteins needed for respiration and gluconeogenesis (DeRisi *et al.* 1997; Schuller 2003; Zitomer and Lowry 1992). Thus the organism can utilize the ethanol that was generated during the fermentative metabolism (DeRisi *et al.* 1997; Schuller 2003; Gancedo 1998). The CCAAT-binding factor (CBF; the Hap2/Hap3/Hap4/Hap5 complex) is one of the transcriptional activators responsible for the activation of many genes involved in respiratory metabolism (DeRisi *et al.* 1997; Schuller 2003; Gancedo 1998; Zitomer and Lowry 1992) as well as other genes needed for the other metabolic functions, such as ammonia assimilation (Dang *et al.* 1996; Riego *et al.* 2002).

Three heterologous subunits of CBF, Hap2/Hap3 and Hap5 are required for DNA binding and this trimer has been shown to be sufficient for CCAAT-specific binding at target promoter (McNabb *et al.* 1995). The fourth subunit of the complex, Hap4 is necessary for the transcriptional activation (Forsburg and Guarente 1989). *HAP4* expression is subject to glucose repression and activated only in the absence of glucose. While the expression of *HAP2*, *HAP3* and *HAP5* is constitutive (DeRisi *et al.* 1997). Thus, the synthesis and interaction of Hap4 with Hap2/Hap3/Hap5 modulate the activity of target genes.

Std1 shows interaction with Hap2 and Hap5 proteins of the CBF complex (*Saccharomyces* Genome Database). This interaction may be in a yet unidentified way

playing a regulatory role in transcriptional activation of some of the respiration genes (*IDP1*, *KGD2*, *SDH1*, *IDH2*, and *QCR2*) identified in the microarray analysis of *mth1Δstd1Δ*.

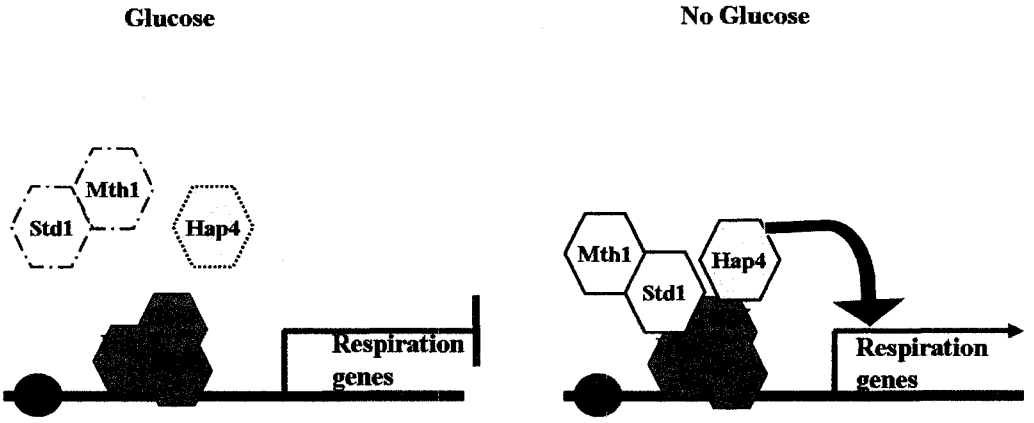


Figure 26. Std1 interacts with Hap2 and Hap5 of CBF complex and activates respiration genes in absence of glucose. The derepression of genes encoding for the components of electron transport chain and tricarboxylic acid (TCA) cycle requires CBF transcriptional activator complex composed of Hap2/Hap3/Hap4 and Hap5 subunits. Hap2/Hap3 and Hap5 bind DNA as trimeric complex and are expressed constitutively. The expression of *HAP4* is repressed is presence of glucose (represented by dashed lined hexagon in glucose). *HAP4* expression is induced in absence of glucose (solid hexagon in no glucose condition). Mth1 and Std1 are degraded in the presence of glucose (represented by broken hexagon in glucose condition). Hap4 interacts with Hap2/Hap3 and Hap5 complex in absence of glucose and activates the expression of respiration genes. Std1 also interacts with Hap2 and Hap5 in no glucose condition. Std1 (probably Mth1) serve as transcriptional regulators and may play a role in derepressing respiration in the absence of glucose.

Microarray analysis identified new functional roles of Mth1 and Std1. Interestingly numerous genes encoding proteins involved in respiration and other mitochondrial functions were identified. Characterizing the mechanism by which Mth1 and Std1 regulate the expression of respiration genes will provide more information for understanding the gene regulation by glucose and other carbon sources. Future experiments needed in this direction include 1) Determining the binding of Rgt1 to the

promoters of respiration genes identified by microarray analysis of *mth1Δstd1Δ*. Majority of the respiration genes identified have one or more Rgt1 binding consensus sites in their promoters. *GND2*, *MSK1*, *ISF1*, *MIC17*, *CIT1*, *FUM1* and *IDH2* show reduced levels of expression in *mth1Δstd1Δ* even though they have Rgt1 binding consensus sites. It is important to find out experimentally if Rgt1 really binds to the promoters in these genes by doing a chromatin immunoprecipitation pull down of Rgt1 in *mth1Δstd1Δ* cells. 2) *IDP1*, *QCR2*, *SDH1* and *KGD2* show reduced levels of expression in *mth1Δstd1Δ* cells and also they do not have Rgt1 binding consensus sites. In this case Mth1 and Std1 may be acting as activators in the absence of glucose to regulate the expression of these genes. Mth1 and Std1 may be serving as activators in conjunction with other transcriptional regulators (like Hap2/3/4/5 transcriptional activation complex). Further characterization is needed to identify the unknown transcriptional factors with which Mth1/Std1 associate in regulating the expression of respiration genes like *IDP1*, *QCR2*, *SDH1* and *KGD2*.

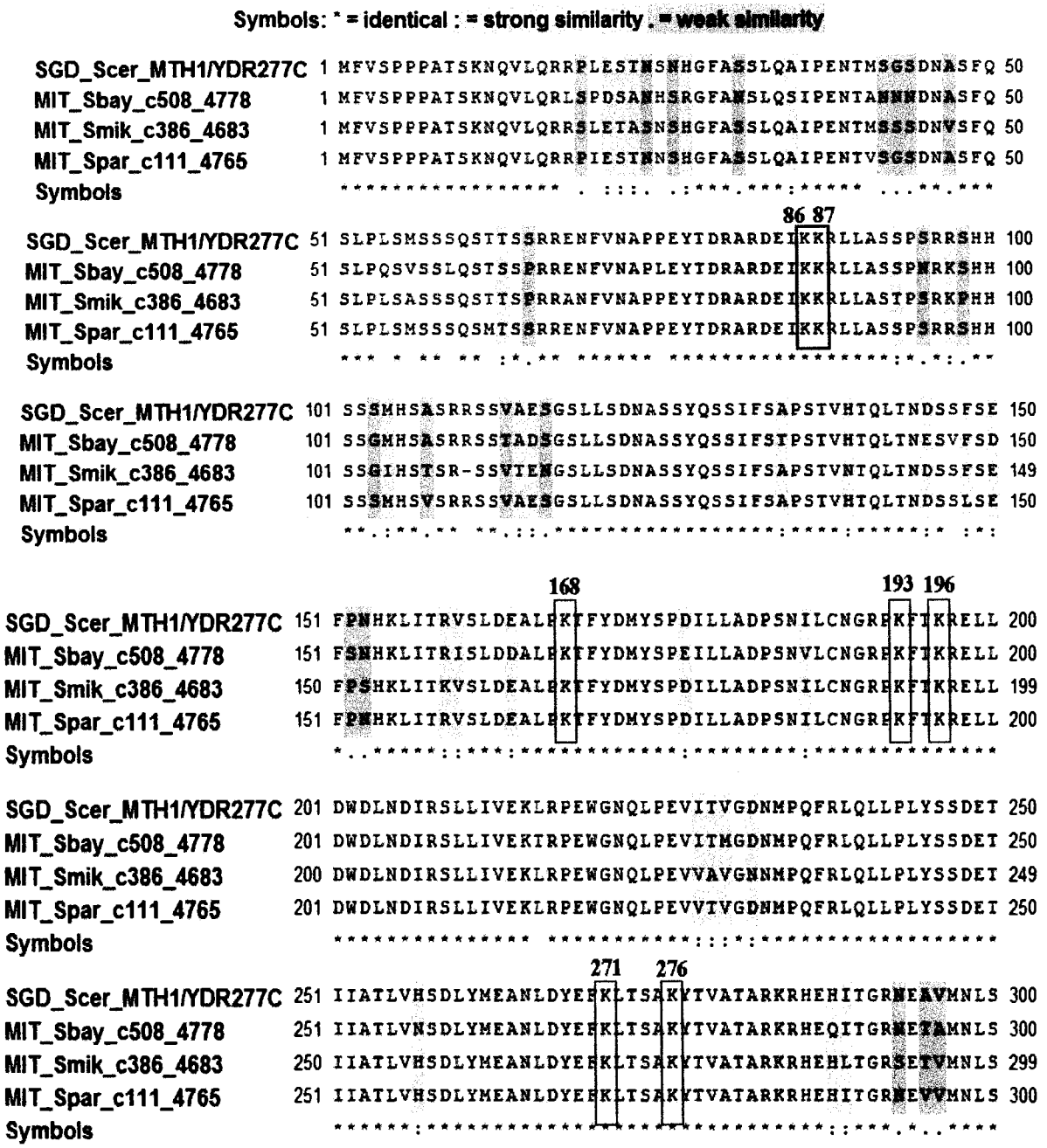
S. cerevisiae prefers glucose as its prime carbon and energy source. This preference is reflected by the variety of glucose-sensing and signaling mechanisms ensuring its optimal use. Glucose is also the prime carbon and energy source in higher multicellular organisms and it is becoming clear that glucose-sensing and signaling in these organisms is of vital importance for maintenance of sugar homeostasis (Rolland *et al.* 2001). In mammals glucose serves as the blood sugar and maintenance of the glucose concentration within narrow limits is controlled by a complex interplay of several endocrine and neural glucostatic systems that direct its uptake and release (Matschinsky *et al.* 1998). Since nutrient-sensing and signaling mechanisms are conserved in wide

variety of organisms, yeast are therefore an excellent model system for studying signal transduction in general.

APPENDIX A1

CLUSTAL W ALIGNMENT OF Mth1 WITH ORTHOLOGS OF OTHER YEAST SPECIES

To identify the conserved lysine residues in Mth1, I used ClustalW alignment feature provided in *Saccharomyces* Genome Database. The program aligns amino acid sequence of protein of interest with other fungal ortholog sequences from Cliften *et al.* 2003 and Kellis *et al.* 2003. The other fungal species protein sequences shown in this alignment include *Saccharomyces bayanus, Saccharomyces mikitae* and *Saccharomyces paradoxus*. Each colors and symbols represent different similarities. Yellow color (and "*" symbol show amino acids which are identical in all the orthologs compared. Pink color (and ":" symbol show strong similarity and green color (and "." Symbol) shows weak similarity in the Mth1 orthologs compared in the indicated fungal species. The rectangle box indicates the conserved lysine with the number of that residue in *S. cerevisiae* displayed on the top of each rectangle.


Symbols: * = identical : = strong similarity . = weak similarity
SGD_Scer_MTH1/YDR277C 1 MFVSPPPATSKNQVLQRRPLESIMSNHGFASSLQAIPENTMSGSDNASFQ 50
MIT_Sbay_c508_4778 1 MFVSPPPATSKNQVLQRLSPDSANHSRGFANSLQSIPENTANNNDNASFQ 50
MIT_Smik_c386_4683 1 MFVSPPPATSKNQVLQRRSLETASNSHGFASSLQAIPENTMSSSDNVSFQ 50
MIT_Spar_c111_4765 1 MFVSPPPATSKNQVLQRRPIESIMNSHGFASSLQAIPENTVSGSDNASFQ 50
Symbols
86 87
SGD_Scer_MTH1/YDR277C 51 SLPLSMSSSQSTTSSRRENFVNAPPEYTDRARDEIKKRLLASSPSRRSHH 100
MIT_Sbay_c508_4778 51 SLPQSVSSLQSTSSPRRENFVNAPLEYTDRARDEIKKRLLASSPNRKSHH 100
MIT_Smik_c386_4683 51 SLPLSASSSQSTTSPRRANFVNAPPEYTDRARDEIKKRLLASTPSRKPHH 100
MIT_Spar_c111_4765 51 SLPLSMSSSQSMTSSRRENFVNAPPEYTDRARDEIKKRLLASSPSRRSHH 100
Symbols
SGD_Scer_MTH1/YDR277C 101 SSSMHSASRRSSVAESGSLLSDNASSYQSSIFSAPSIVHTQLTNDSSFSE 150
MIT_Sbay_c508_4778 101 SSGMHSASRRSSTADSGSLLSDNASSYQSSIFSIPSIVHTQLTNESVFSD 150
MIT_Smik_c386_4683 101 SSGIHSTSR-SSVTENGSLLSDNASSYQSSIFSAPSIVNTQLTNDSSFSE 149
MIT_Spar_c111_4765 101 SSSMHSVSRRSSVAESGSLLSDNASSYQSSIFSAPSIVHTQLTNDSSLSE 150
Symbols
168
193 196
SGD_Scer_MTH1/YDR277C 151 FPNHKLITRVSLDEALEKTFYDMYSPDILLADPSNILCNGREKFIKRELL 200
MIT_Sbay_c508_4778 151 FSNHKLITRISLDDALEKTFYDMYSPEILLADPSNVLCNGREKFIKRELL 200
MIT_Smik_c386_4683 150 FPSHKLITKVSLDEALEKTFYDMYSPDILLADPSNILCNGREKFIKRELL 199
MIT_Spar_c111_4765 151 FPNHKLITRVSLDEALEKTFYDMYSPDILLADPSNILCNGREKFIKRELL 200
Symbols
SGD_Scer_MTH1/YDR277C 201 DWDLNDIRSLLIVEKLRPEWGNQLPEVITVGDNMPQFRLQLLPLYSSDET 250
MIT_Sbay_c508_4778 201 DWDLNDIRSLLIVEKIRPEWGNQLPEVITMGDNMPQFRLQLLPLYSSDET 250
MIT_Smik_c386_4683 200 DWDLNDIRSLLIVEKLRPEWGNQLPEVVAVGNNMPQFRLQLLPLYSSDET 249
MIT_Spar_c111_4765 201 DWDLNDIRSLLIVEKLRPEWGNQLPEVVTVGDNMPQFRLQLLPLYSSDET 250
Symbols
271 276
SGD_Scer_MTH1/YDR277C 251 IIATLVHSDLYMEANLDYEEKLTSAKYTVATARKRHEHITGRMEAVMNLS 300
MIT_Sbay_c508_4778 251 IIATLVNSDLYMEANLDYEEKLTSAKYTVATARKRHEQITGRMETAMNLS 300
MIT_Smik_c386_4683 250 IIATLVHSDLYMEANLDYEEKLTSAKYTVATARKRHEHLTGRSETVMNLS 299
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Symbols

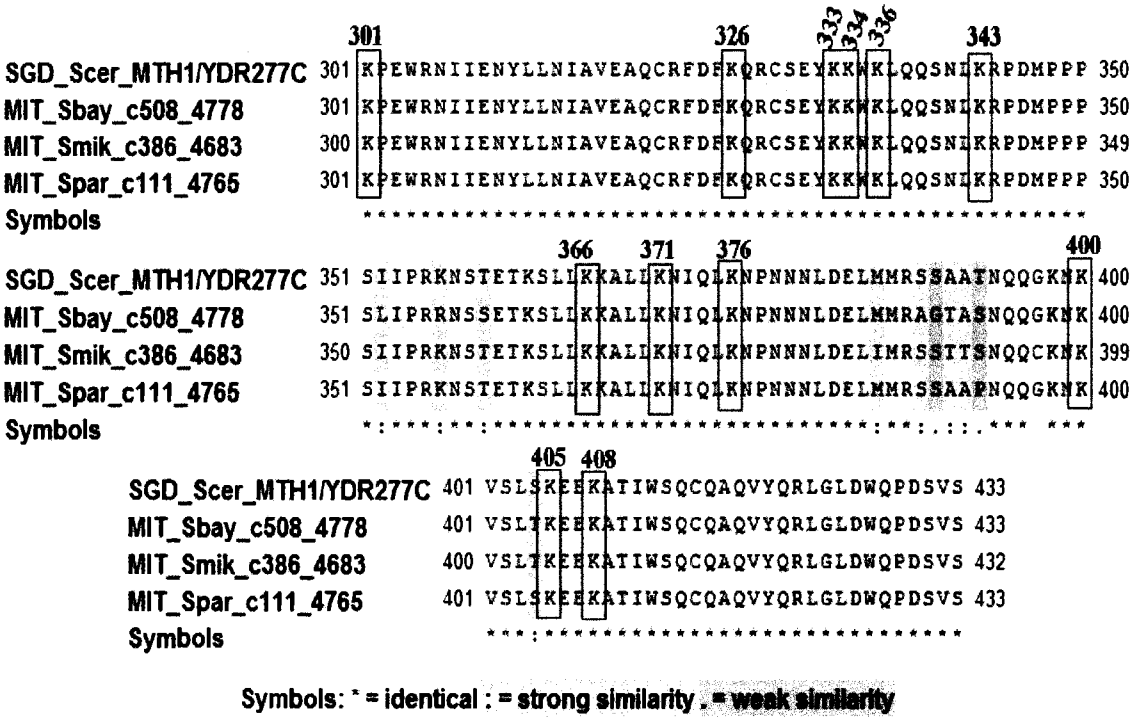
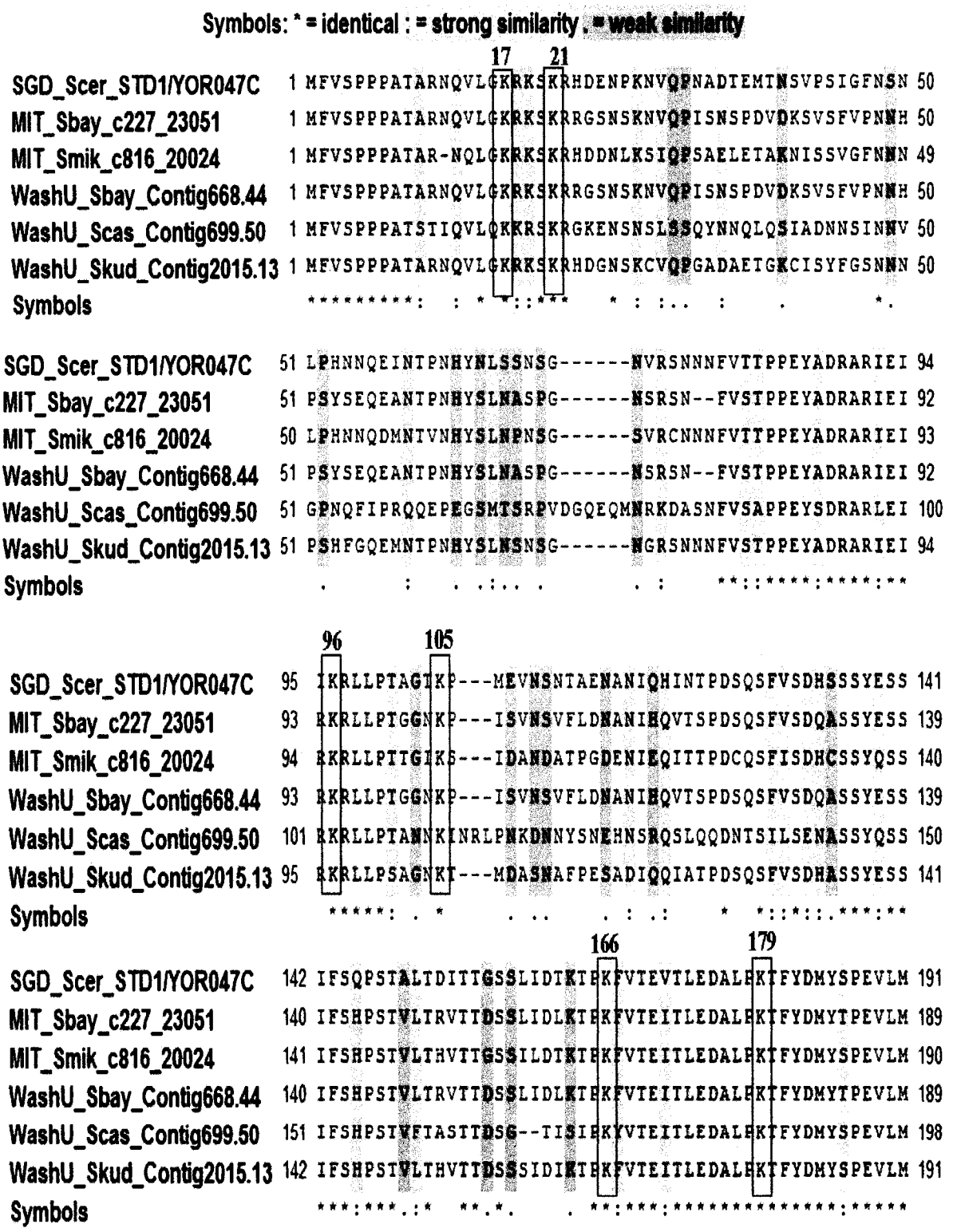


Figure 27. Clustal W protein alignment of Mth1 shows conserved regions in its orthologs from other yeast species.

APPENDIX A2

CLUSTALW ALIGNMENT OF Std1 WITH ORTHOLOGS OF OTHER YEAST SPECIES

To identify the conserved lysine residues in Std1, I used ClustalW alignment feature provided in *Saccharomyces* Genome Database. The program aligns amino acid sequence of protein of interest with other fungal ortholog sequences from Cliften *et al.* 2003 and Kellis *et al.* 2003. The other fungal species protein sequences shown in this alignment include *Saccharomyces bayanus, Saccharomyces mikitae* and *Saccharomyces castellii* and *Saccharomyces krudriavzevii*. Each colors and symbols represent different similarities. Yellow color (and "*" symbol show amino acids which are identical in all the orthologs compared. Pink color (and ":" symbol show strong similarity and green color (and "." Symbol) shows weak similarity in the Mth1 orthologs compared in the indicated fungal species. The rectangle box indicates the conserved lysine with the number of that residue in *S. cerevisiae* displayed on the top of each rectangle.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21
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WashU_Skud_Contig2015.13 1 MFVSPPPATARNQVLGKRKSKRHDGNSKCVQPGADAETGKCISYFGSNNN 50
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MIT_Smik_c816_20024 50 LPHNNQDMNTVNHYSLNPNSG------SVRCNNNFVTTPPEYADRARIEI 93
WashU_Sbay_Contig668.44 51 PSYSEQEANTPNHYSLNASPG------NSRSN--FVSTPPEYADRARIEI 92
WashU_Scas_Contig699.50 51 GPNQFIPRQQEPEGSMTSRPVDGQEQMNRKDASNFVSAPPEYSDRARLEI 100
WashU_Skud_Contig2015.13 51 PSHFGQEMNTPNHYSLNSNSG------NGRSNNNFVSTPPEYADRARIEI 94
Symbols
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MIT_Sbay_c227_23051 93 RKRLLPTGGNKP---ISVNSVFLDNANIHQVTSPDSQSFVSDQASSYESS 139
MIT_Smik_c816_20024 94 RKRLLPTIGIKS---IDANDATPGDENIEQITTPDCQSFISDHCSSYQSS 140
WashU_Sbay_Contig668.44 93 RKRLLPTGGNKP---ISVNSVFLDNANIHQVTSPDSQSFVSDQASSYESS 139
WashU_Scas_Contig699.50 101 RKRLLPTANNKINRLPNKDNNYSNEHNSRQSLQQDNTSILSENASSYQSS 150
WashU_Skud_Contig2015.13 95 RKRLLPSAGNKT---MDASNAFPESADIQQIATPDSQSFVSDHASSYESS 141
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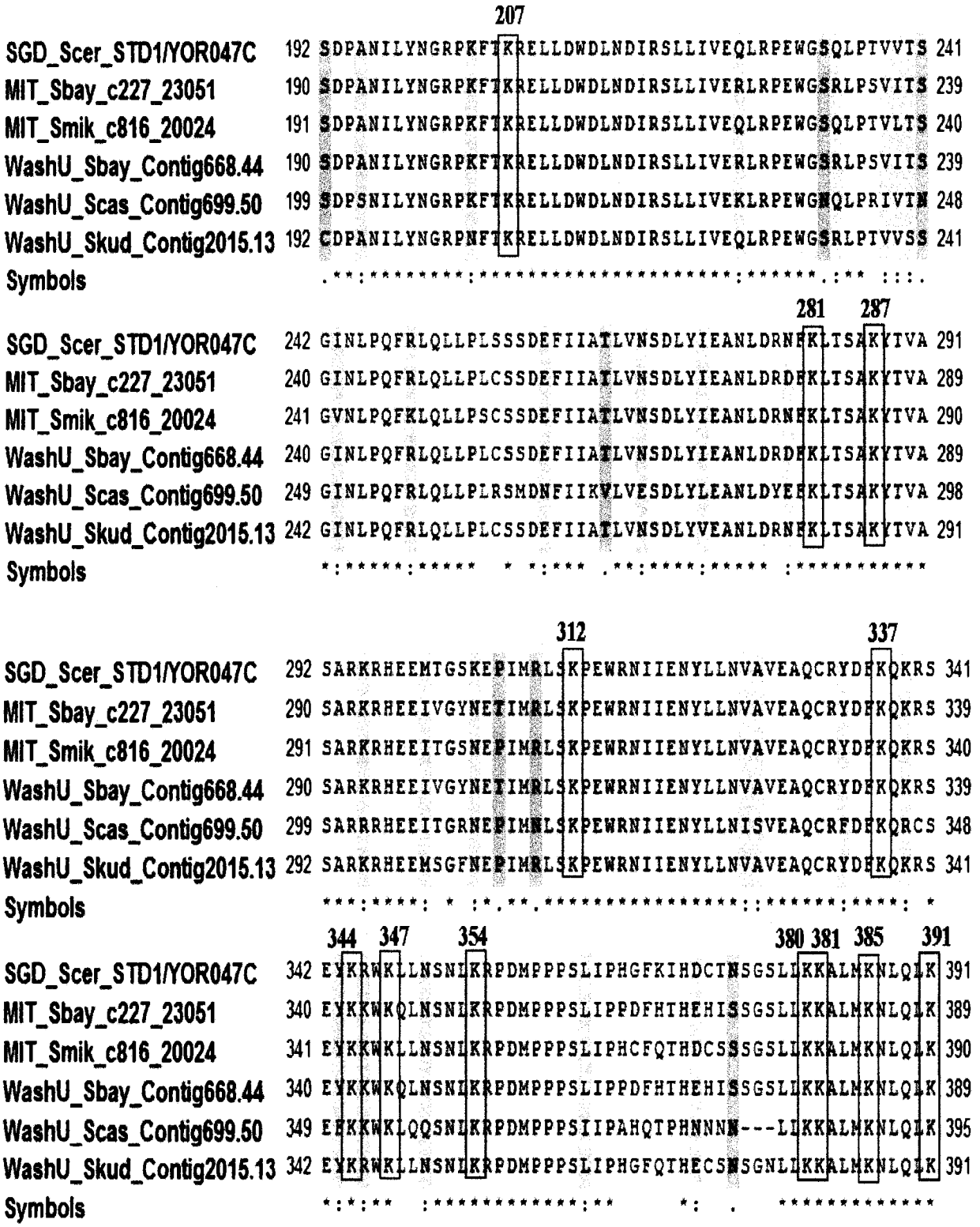
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WashU_Skud_Contig2015.13 242 GINLPQFRLQLLPLCSSDEFIIATLVNSDLYVEANLDRNFKLTSAKYTVA 291
Symbols
312 337
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MIT_Sbay_c227_23051 290 SARKRHEEIVGYNETIMRLSKPEWRNIIENYLLNVAVEAQCRYDFKQKRS 339
MIT_Smik_c816_20024 291 SARKRHEEITGSNEPIMRLSKPEWRNIIENYLLNVAVEAQCRYDFKQKRS 340
WashU_Sbay_Contig668.44 290 SARKRHEEIVGYNETIMRLSKPEWRNIIENYLLNVAVEAQCRYDFKQKRS 339
WashU_Scas_Contig699.50 299 SARRRHEEITGRNEPIMRLSKPEWRNIIENYLLNISVEAQCRFDFKQRCS 348
WashU_Skud_Contig2015.13 292 SARKRHEEMSGFNEPIMRLSKPEWRNIIENYLLNVAVEAQCRYDFKQKRS 341
Symbols
344 347 354 380 381 385 391
SGD_Scer_STD1/YOR047C 342 EYKRWKLLNSNLKRPDMPPPSLIPHGFKIHDCINSGSLLKKALMKNLQLK 391
MIT_Sbay_c227_23051 340 EYKKWKQLNSNLKRPDMPPPSLIPPDFHTHEHISSGSLLKKALMKNLQLK 389
MIT_Smik_c816_20024 341 EYKKWKLLNSNLKRPDMPPPSLIPHCFQTHDCSSSGSLLKKALMKNLQLK 390
WashU_Sbay_Contig668.44 340 EYKKWKQLNSNLKRPDMPPPSLIPPDFHTHEHISSGSLLKKALMKNLQLK 389
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Symbols

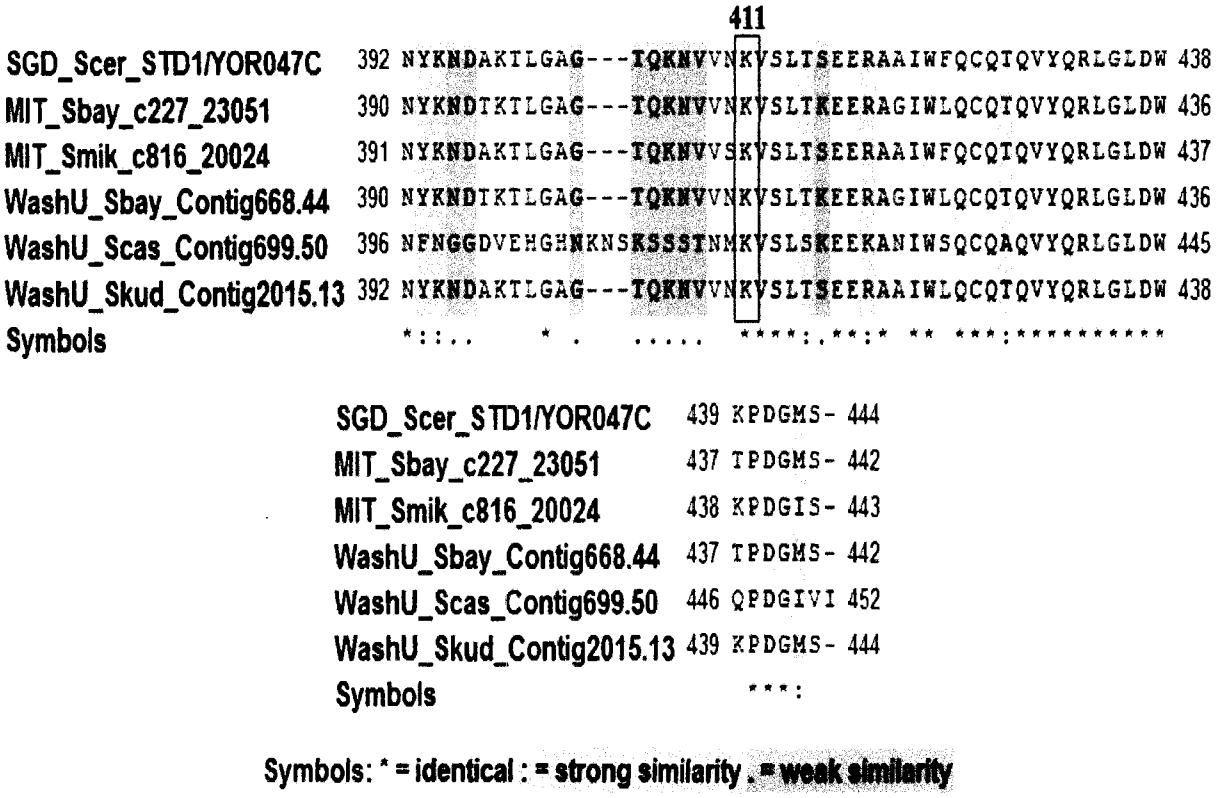

Figure 28. Clustal W protein alignment of Std1 shows conserved regions in its orthologs from other yeast species

APPENDIX B

ANALYSIS OF Rgt1-DNA BINDING SITES IN INDUCED AND REPRESSED GENES IDENTIFIED BY TRANSCRIPTOMIC ANALYSIS OF *mth1Δstd1Δ*

The following tables lists the down-regulated (induced by Mth1 and Std1) and up-regulated (repressed by Mth1 and Std1) genes in *mth1Δstd1Δ* cells relative to isogenic wild type cells during the steady state growth in YEP + 2% galactose medium. Genes are listed by their systemic ORF ID (ORF column) followed by common gene name (Gene). Column Rgt1 site, indicates the presence "Y" or absence "N" of Rgt1-DNA binding consensus sites $_{5'}$CGGANNA$_{3'}$ in the promoters of the corresponding genes. The next column lists the number of Rgt1-DNA binding consensus sites. Aneta *et al.* column lists whether the gene was identified in the microarray analysis done with *rgt1Δ* cells in galactose medium. The last column (Essential) indicates if the corresponding gene is essential or not for the survival of the *S. cerevisiae* cells.

GENES INDUCED BY Mth1 AND Std1

ORF	*Gene*	Galactose	Rgt1 site	No. of Rgt1 sites	Aneta et al. *rgt1Δ*	Essential
YAL003W	*EFB1*	-1.6	Y	1	N	Y
YAL046C		-1.0	Y	1	N	N
YBR083W	*TEC1*	-1.36	Y	1	N	N
YCL027C-A		-1.2	N	0	N	N
YCR004C	*YCP4*	-1.14	Y	1	N	N
YDL066W	*IDP1*	-1.35	N	0	N	N
YDL079C	*MRK1*	-1.85	Y	2	N	N
YDR148C	*KGD2*	-1.74	N	0	N	N
YDR154C		-1.45	N	0	N	N
YDR277C	*MTH1*	-3.16	N	0	Y	N
YDR321W	*ASP1*	-1.2	Y	4	N	Y
YER003C	*PMI40*	-1.11	Y	3	N	Y
YER043C	*SAH1*	-1.5	N	0	N	Y
YER138W-A		-1.4	N	0	N	N
YFL026W	*STE2*	-1.7	Y	1	N	N
YGL040C	*HEM2*	-1.1	N	0	N	Y
YGL105W	*ARC1*	-1.38	Y	2	N	N
YGR136W	*LSB1*	-1.08	Y	1	N	N
YGR137W		-1.36	Y	2	N	N
YGR256W	*GND2*	-1.58	Y	3	N	N
YHR008C	*SOD2*	-1.5	Y	2	N	N
YHR033W		-1.2	N	0	N	N
YIL015W	*BAR1*	-1.29	N	0	N	N
YIL041W	*GVP1*	-1.59	N	0	N	N
YIL070C	*MAM33*	-1.2	N	0	N	N
YJL026W	*RNR2*	-1.3	Y	1	N	Y
YJL130C	*URA2*	-1.6	Y	2	N	N
YJL170C	*ASG7*	-1.3	N	0	N	N
YJR007W	*SUI2*	-1.1	Y	2	N	Y
YKL085W	*MDH1*	-1.4	N	0	N	N
YKL096W	*CWP1*	-1.2	N	0	N	N
YKL216W	*URA1*	-1.4	Y	1	N	N
YLR048W	*RPS0B*	-1.18	Y	1	N	N
YLR150W	*STM1*	-1.3	Y	2	N	N
YLR172C	*DPH5*	-1.46	Y	3	N	N
YLR178C	*TFS1*	-1.4	Y	1	N	N

YLR293C	*GSP1*	-1.3	Y	1	N	N
YLR304C	*ACO1*	-1.2	Y	3	N	N
YLR354C	*TAL1*	-1.7	Y	1	N	N
YLR356W		-1.0	Y	3	N	N
YLR448W	*RPL6B*	-1.37	N	0	N	N
YML126C	*ERG13*	-1.32	Y	4	N	Y
YMR002W	*MIC17*	-1.1	Y	2	N	N
YMR058W	*FET3*	-1.4	Y	1	N	N
YMR081C	*ISF1*	-1.6	Y	2	N	N
YMR186W	*HSC82*	-1.4	N	0	N	N
YNL037C	*IDH1*	-1.2	Y	3	N	N
YNL055C	*POR1*	-1.9	N	0	N	N
YNL073W	*MSK1*	-1.16	Y	1	N	N
YNL160W	*YGP1*	-1.59	Y	2	N	N
YNL209W	*SSB2*	-1.3	Y	1	N	N
YOL040C	*RPS15*	-1.46	Y	7	N	Y
YOL101C	*IZH4*	-1.1	Y	4	N	N
YOL126C	*MDH2*	-1.5	Y	6	N	N
YOL143C	*RIB4*	-1.2	N	0	N	N
YOR136W	*IDH2*	-1.63	Y	1	N	N
YOR185C	*GSP2*	-1.4	Y	2	N	N
YOR224C	*RPB8*	-1.2	N	0	N	Y
YPL066W		-1.5	Y	4	N	N
YPL131W	*RPL5*	-1.4	N	0	N	Y
YPR043W	*RPL43A*	-1.8	Y	2	N	N
YPR044C	*OPI11*	-1.1	Y	1	N	N
YPR063C		-1.5	N	0	N	N
YPR144C	*UTP19*	-1.1	N	0	N	Y
YPR191W	*QCR2*	-1.38	N	0	N	N

GENES REPRESSESED BY Mth1 AND Std1

ORF	*Gene*	Galactose	Rgt1 site	No. of Rgt1 sites	Aneta et al. *rgt1Δ*	Essential
YBL048W		1.4	Y	1	N	N
YBL049W	*MOH1*	1.3	Y	1	N	N
YBR104W	*YMC2*	1.12	N	0	N	N
YCL030C	*HIS4*	1.45	Y	1	N	N
YDR342C	*HXT7*	1.9	Y	Multiple	Y	N
YDR343C	*HXT6*	1.5	Y	Multiple	Y	N
YDR345C	*HXT3*	7.0	Y	Multiple	Y	N
YER028C	*MIG3*	1.4	Y	5	Y	N
YER037W	*PHM8*	1.4	N	0	Y	N
YGL009C	*LEU1*	2.7	Y	1	N	N
YGL157W		2.87	N	0	Y	N
YGL184C	*STR3*	1.1	Y	2	N	N
YGL209W	*MIG2*	3.76	Y	Multiple	Y	N
YGL258W	*VEL1*	1.4	Y	1	N	N
YHR092C	*HXT4*	4.3	Y	Multiple	Y	N
YHR094C	*HXT1*	6.25	Y	Multiple	Y	N
YJR137C	*ECM17*	1.56	Y	1	N	N
YKL001C	*MET14*	1.5	N	0	N	N
YKL120W	*OAC1*	2.0	Y	3	N	N
YKR075C		2.8	N	0	Y	N
YLR108C		1.8	Y	2	N	N
YLR130C	*ZRT2*	1.38	Y	2	N	N
YMR011W	*HXT2*	3.5	Y	Multiple	Y	N
YMR096W	*SNZ1*	1.8	Y	4	N	N
YMR319C	*FET4*	1.5	Y	2	N	N
YNL234W		1.6	Y	2	Y	N
YOR306C	*MCH5*	1.7	Y	3	N	N
YOR387C		2.0	N	0	N	N

APPENDIX C

PRIMERS FOR RT-PCR VALIDATION OF MICROARRAY ANALYSIS OF *mth1Δstd1Δ* MUTANT CELLS

Primer[a]	Systematic Name	Sequence (5'→3')	Reference
ACT1 for	YFL039C	GGTTATTGATAACGGTTCTGGTATG	Brickner *et al.* 2007
ACT1 rev	YFL039C	ATGATACCTTGGTGTCTTGGTCTAC	Brickner *et al.* 2007
ASP1 for	YDR312W	CTGCATGAAATTGAGTTTGC	This Study
ASP1 rev	YDR312W	TCCTTTGAACCTTTCTTTGG	This Study
URA1 for	YKL216W	TTATCCTGATGCACCTGCTA	This Study
URA1 rev	YKL216W	TTCGCTATCTTGGATTTTCC	This Study
URA2 for	YJL130C	GGTAGGCAATTATGGTGTCC	This Study
URA2 rev	YJL130C	AACCGGCAATATGGATTCTA	This Study
SAH1 for	YER043C	ACCACAAGTCGACCGTTACT	This Study
SAH1 rev	YER043C	ACCAGTAGCACAACCCAAGT	This Study
HIS4 for	YCL030C	TGGTAAATTCAGCGATGATG	This Study
HIS4 rev	YCL030C	GTTCGGCTGTTTTAGCATCT	This Study
LEU1 for	YGL009C	GTTTTATGAATGGCGTTCC	This Study
LEU1 rev	YGL009C	TATTTCAGCTTCCCTCCAAG	This Study
MET14 for	YKL001C	AGGCATTGAGAAAACAGGAC	This Study
MET14 rev	YKL001C	TAACTGTTCTAGCGCACAGG	This Study
STR3 for	YGL184C	CAACCATACGCCAACAATAA	This Study
STR3 rev	YGL184C	GACTGCATGACTCTGTTGCT	This Study
ECM17 for	YJR137C	GTCTTTCTGGGAAGCTCTGA	This Study
ECM17 rev	YJR137C	GGCCAATACTCAGAATCACC	This Study
GND2 for	YGR256W	GGACGCAATCAAAGGTAGAT	This Study
GND2 rev	YGR256W	TAGTTTCGCAACCAAGTCCT	This Study
SOD2 for	YHR008C	CAAGGAGAACCAAAGTCACC	This Study
SOD2 rev	YHR008C	GGTGTAATGCAATTCGTTGA	This Study
MSK1 for	YNL073W	TCCGTCAATGTCAGAATCAG	This Study
MSK1 rev	YNL073W	GTTGCCCAAGAATCTCTCAT	This Study
TAL1 for	YLR354C	ATGGTAAGACCACCGAAGAA	This Study

TAL1 rev	YLR354C	CTCTGCCTGGAACAATCTTT	This Study
ISF1 for	YMR081C	AATCCTTTTCGCTTCTTTCC	This Study
ISF1 rev	YMR081C	ATTGGAAGGAGATTTCACCA	This Study
QCR2 for	YPR191W	AGACTGCCTTCAAACCTCAC	This Study
QCR2 rev	YPR191W	GCTCTTTACCGGACATTGTT	This Study
MIC17 for	YMR002W	ACGAGACAGCCAGGTATGTT	This Study
MIC17 rev	YMR002W	TACCGGTAATACCTGCACCT	This Study
YCP4 for	YCR004C	GATGAACGCTCCTCAGAAAC	This Study
YCP4 rev	YCR004C	CCAAACCTAGTTGGAACACC	This Study
IDP1 for	YDL066W	TGATGAAGCTCGTGTGAAGG	This Study
IDP1 rev	YDL066W	CTGTACCGCCGAGAATGTTT	This Study
ACO1 for	YLR304C	TGTCAGAGTCGGTTTGATCG	This Study
ACO1 rev	YLR304C	TTTCAAACCATGAGCAGCAG	This Study
CIT1 for	YNR001C	ACGTTGAAGGAGAGATTTGC	This Study
CIT1 rev	YNR001C	CGGTTTTACCGTGTTCTTTC	This Study
CIT2 for	YCR005C	GCTAAGGCTTATGCTCAAGG	This Study
CIT2 rev	YCR005C	GCAATTTACCCAGCAAGTCT	This Study
HXT1 for	YHR094C	CTGGTTTAGGTGTCGGTGGT	This Study
HXT1 rev	YHR094C	CATGAAACCAAGGTGCCTCT	This Study
FUM1 for	YPL262W	CACCCTTGCTTGTTCTCTTT	This Study
FUM1 rev	YPL262W	TTCATTCTCTGGCAGCATTA	This Study
SDH1 for	YKL148C	AGTACTTTGCCCTCGATCTG	This Study
SDH1 rev	YKL148C	GTGTGCTCTGAATCTGTGGA	This Study
KGD2 for	YDR148C	AGGCTGATCAACCAAAGAAG	This Study
KGD2 rev	YDR148C	TTCTGTACGTGGAAATGGTG	This Study
IDH2 for	YOR136W	CAAATACACGGTCTCGTTCA	This Study
IDH2 rev	YOR136W	GACGTTTGCTGCACTAAAGA	This Study
MCH5 for	YOR306C	GCACGACAGTGACAAAGAAG	This Study
MCH5 rev	YOR306C	GAAGCAACCAAATGTCACAA	This Study
FET3 for	YMR058W	AACGTTGATGGGCTAAAGAG	This Study
FET3 rev	YMR058W	AGTAAATCTGCACACGGTCA	This Study
ZRT2 for	YLR130C	TATCGTCAGGATTGGGAGTT	This Study
ZRT2 rev	YLR130C	AACCGAAGAACTTCGCTATG	This Study
OAC1 for	YKL120W	CAAATTGGGCTAAATGGTTC	This Study
OAC1 rev	YKL120W	TTATGTGGCTCTTGATCTGG	This Study

FET4 for	YMR319C	ATGTTTGGGACACACTGTTG	This Study
FET4 rev	YMR319C	AAGCCAATCTCGACTTCAAT	This Study
YMC2 for	YBR104W	ATCGGTGGTATTGCACAAGT	This Study
YMC2 rev	YBR104W	ACCTCCAACGTTGTTGTTCT	This Study
MRK1 for	YDL079C	GTTGTGCCATCCAAATACTG	This Study
MRK1 rev	YDL079C	GAGGCATGTAGTCCAAAACC	This Study

Primer[a] for = forward primer and rev = reverse primer

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