POST-TRANSCRIPTIONAL ROLE OF *SNF1* PROTEIN KINASE IN MALTOSE PERMEASE SYNTHESIS OF *SACCHAROMYCES CEREVISIAE*

by

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A dissertation submitted to the Graduate Faculty in Biology in partial fulfillment of the requirements for the degree of Doctor of Philosophy,
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ABSTRACT

POST-TRANSCRIPTIONAL ROLE OF *SNF1* PROTEIN KINASE IN MALTOSE PERMEASE SYNTHESIS OF *SACCHAROMYCES CEREVISIAE*

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Saima Arshad Cheema

Adviser: Professor Corinne A. Michels

Loss of Snf1 kinase blocks *MAL* gene induction even if *MIG1* is also deleted (Hu et al., 2000). Here I show that this defect in *MAL* gene induction stems from defective maltose permease expression. This dissertation investigates the post-transcriptional requirement of Snf1 kinase in maltose permease synthesis.

Expression of a series of *MAL61-LacZ* fusions with junction sites in the *MAL61* ORF reveals that *MAL61* mRNA usage is only modestly affected by loss of *SNF1*. However, Mal61/HA protein levels are dramatically reduced in a *snf1Δ mig1Δ* strain even when *MAL61/HA* is transcribed from the constitutive GPD promoter indicating that maltose permease synthesis requires *SNF1* at a post-transcriptional step. *GAL2, HXT1* and *MAL61*, all members of the 12TMD family of sugar transporters, were fused to the GPD promoter and, in contrast to Mal61p, synthesis of Hxt1p and Gal2p and galactose transport activity are unaffected by *snf1Δ*. Various hybrid *MAL61-GAL2* and *GAL2-MAL61* genes were constructed and their expression in a *snf1Δ mig1Δ* strain indicates that the *SNF1* requirement for the Mal61p expression is associated with its N-terminal half.

UPR and ERAD are protein quality control pathways involved in the degradation of improperly folded/denatured ER-localized proteins. In the absence of several key components of either UPR or ERAD, particularly Ire1p, Ubc6p, and Doa10p, Mal61p is stabilized suggesting that Mal61p becomes a target of these degradation pathways in the absence of *SNF1*. Additionally, Mal61p levels increase significantly in a *snf1Δ mig1Δ end3Δ* strain but this is not associated with an increase in transport activity indicating that permease is rapidly internalized in the *snf1Δ* strain and that the protein that reaches the plasma membrane is inactive. Consistent with this, Mal61p levels become stable in the absence of *PEP4*.

Finally, multicopy suppressors of the maltose non-fermenting phenotype of the *snf1Δ mig1Δ* [pMAL63/43-c] strain were isolated and characterized.

The results show that Snf1 kinase is required post-transcriptionally at several steps in maltose permease expression. In the absence of *SNF1*, maltose permease is a target of the ER quality control pathways, is rapidly internalized from the plasma membrane, and is functionally inactive.

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INTRODUCTION

For baker's yeast, *Saccharomyces cerevisiae*, glucose is the preferred carbon source. A major route by which glucose promotes its own use and stimulates fermentation is by regulating gene expression. To accomplish this, yeast has evolved numerous mechanisms that act at the levels of gene transcription, mRNA stability, mRNA translation and protein stability (Johnston, 1999). The major down regulating effect takes place at the level of transcription. Various classes of genes that are repressed by glucose include those that encode proteins involved in respiration, gluconeogenesis, and metabolism of alternative carbon sources such as the *GAL*, *SUC* and *MAL* genes. A large group of STRE-controlled genes encoding proteins involved in the yeast's response to various stresses are repressed by glucose (Rolland *et al.*, 2002). Transcription of the glucose-repressible genes in derepressing conditions is dependent on the Snf1-protein kinase. Snf1 kinase is inactive in cells that are rapidly metabolizing glucose and is active in cells growing on alternate carbon sources. In addition to genes for maltose (*MAL*), sucrose (*SUC*) and galactose (*GAL*) utilization, Snf1 kinase is required for transcription of genes involved in mitochondrial biogenesis, meiosis and sporulation, glycogen storage, thermotolerance and other processes (Carlson, 1999).

In *Saccharomyces cerevisiae*, glucose regulates *MAL* gene expression by multiple mechanisms at both the transcription and post-transcriptional levels. Hu et al. (1995) demonstrated that Mig1 repressor binds to sites in *MAL61*, *62* and *63* promoters and blocks transcription in the presence of glucose. Snf1 kinase

negatively regulates Mig1 repressor and thereby releases the *MAL* genes from glucose repression of transcription (Treitel et al., 1998). Disruption of *MIG1* and its homologue *MIG2* fully relieves glucose repression of *MAL* gene expression however induction by maltose is still blocked by glucose (Hu et al., 2000). In results to be described below in detail, Hu et al. (2000) demonstrated that Snf1p, in addition to being required for inactivation of Mig1p, is needed at a post-transcriptional step for the synthesis of maltose permease. This study will explore the role of Snf1 in the post-transcriptional regulation of maltose permease synthesis.

Maltose fermentation and MAL loci

Saccharomyces cerevisiae requires three proteins in order to metabolize maltose. These include maltose permease to transport maltose across the plasma membrane; maltase to breakdown maltose into two glucose molecules; and the *MAL*-activator, a DNA-binding transcription activator to mediate transcription of the three genes including the activator itself. All three of these proteins are products of genes clustered at one of the five unlinked, telomere-associated *MAL* loci: *MAL1, 2, 3, 4* and *6* (Needleman and Michels, 1983). Any one of these complex loci is sufficient to ferment maltose; however, most naturally occurring *Saccharomyces* strains contain more than one locus. Genes encoding maltose permease, maltase, and the *MAL*-activator are present at each locus and all three functions are essential for rapid utilization of maltose (Charron et al., 1989; Needleman et al., 1984). Gene 1 at each locus codes for the

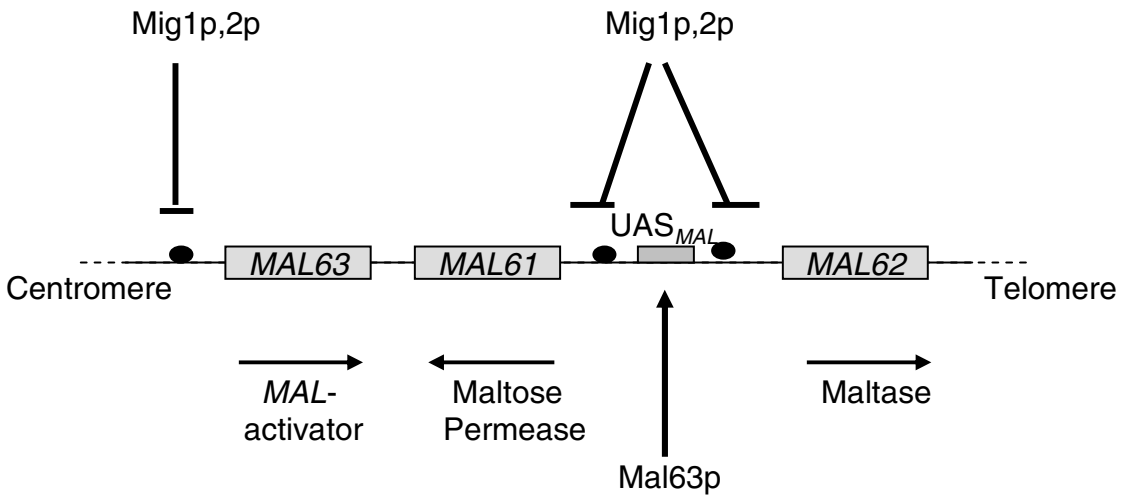
maltose permease protein, gene 2 encodes the maltase protein and gene 3 codes for the transacting *MAL*-activator protein (Needleman, 1991). A two digit numbering system distinguishes each of these genes at various loci where the first digit indicates the locus of the gene and the second digit indicates the gene function. At *MAL6* these genes are called *MAL61, MAL62,* and *MAL63*, respectively (Needleman and Michels, 1983).

The organization and structure of *MAL6* locus is shown in Figure1. *MAL61* and *MAL62*, encoding maltose permease and maltase are transcribed bi-directionally from a common promoter located in the 874 bp sequence separating the two genes (Needleman et al., 1984) where -67 and -843 are transcription start sites for *MAL61* and *MAL62* respectively (Hong and Marmur, 1987). A 68-bp sequence from -515 to -582 of *MAL61* start codon, in the *MAL61-MAL62* intergenic region contains the UAS_{MAL}, the cis-acting regulatory sequence and *MAL*-activator binding site necessary for maltose-induced expression of both genes (Levine et al., 1992). Consequently, the transcription of both genes is coordinately regulated. There is one *MAL*-activator binding site in the promoter of the *MAL63* gene that codes for the *MAL*-activator protein itself.

Maltose permease encoded by *MAL61* and its homologues at the other *MAL* loci, is a high-affinity maltose/proton symporter which uses the energy stored in the H^+ ion electrochemical gradient to move maltose against concentration gradient. It is a member of the 12-transmembrane-domain family of sugar transporters with homology to other high-affinity sugar transporters (Cheng and Michels, 1989; Cheng and Michels, 1991). The structure of Mal61p

Figure1. Organization of *MAL6* locus

At the *MAL6* locus, all three genes required for maltose uptake and utilization are present. *MAL61* and *MAL62* are transcribed from a common bidirectional promoter. Mig1 and Mig2 repressor binding sites are shown along with the upstream activating sequence UAS_{MAL}

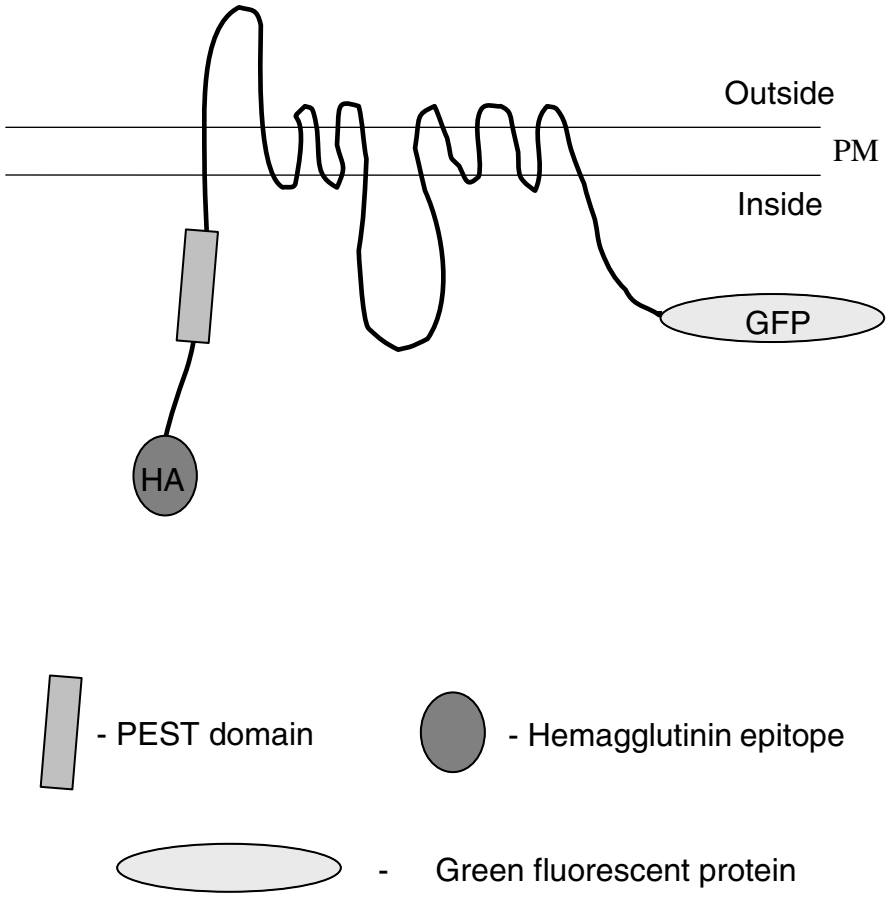


consists of two blocks of six transmembrane domains separated by a 71 residue intracellular region (Figure 2). Both the N-terminal and C-terminal domains of 100 and 70 residues are predicted to lie on the cytoplasmic face of the plasma membrane (Cheng and Michels, 1989). Although two putative N-glycosylation sites, Asn-15 and Asn-27, exist in the N-terminal region, Mal61p has not been shown to be glycosylated. Gadura and Michels (2006) found that the N-terminal cytoplasmic domain contains signal sequences involved in a late step in endocytosis. A putative PEST sequence (rich in proline, aspartate, glutamate, serine and threonine residues) is present in residues 47-78 and deletion of this sequence makes Mal61p resistant to glucose-induced proteolysis (Medintz et al., 2000). The proposal that this PEST sequence is involved in the glucose regulated/induced phosphorylation, ubiquitination, and degradation, was not supported by studies of Gadura and Michels (2006). They showed that mutations of serines/threonines at residues 29, 33, 43, 48, 56 or a dileucine motif at residues 61-64 does not alter Mal61p trafficking to the plasma membrane or its glucose induced internalization. They demonstrated that a late step in endocytosis is altered affecting movement to the vacuole and degradation. They also showed that the yeast casein kinases Yck1 and Yck2 are essential for glucose induced inactivation of the permease (Gadura et al., 2006).

Maltase is an α-glucosidase responsible for hydrolyzing maltose into two molecules of glucose, which are converted to CO_2, ethanol and ATP during glycolysis followed by anaerobic respiration.

Figure 2. The structure of maltose permease

Mal61 maltose permease is 12 transmembrane domain (TMD) family of sugar transporters. The N-terminal PEST (rich in Proline, Aspartate, Glutamate, serine and Threonine) domain is shown along with the N-terminal HA-epitope and C-terminal GFP fusion sites.



The *MAL*-activator, a C6 Zn^{+} finger protein, is a 470-residue DNA-binding transcription activator (Hu et al., 1999) The N-terminal 100 residues constitute the cysteine-rich DNA binding domain. The transactivation domain lies in the mid-region of the protein and spans approximately residues 100-250. The C-terminal 200 residues compose a negative regulatory region that responds to maltose (Hu et al., 1999). Constitutivety in *MAL*-activator results from multiple alterations in this regulatory domain (Danzi et al., 2000; Gibson et al., 1997). The C-terminal regulatory domain also has a positive role in induction and mutations at sites throughout the domain are non-inducible. These results suggest that complex protein-protein interactions and folding are involved in *MAL*-activator induction. This conclusion is supported by the finding that the *MAL*-activator is an Hsp90 client protein (Bali et al., 2003). The Hsp90 molecular chaperone complex is required for maltose induction of the *MAL*-activator and *MAL*-activator protein is found in association with Hsp90 protein, as shown by co-immunoprecipitation.

The induction of *MAL* genes requires an inducer, typically maltose; however, the mechanism of maltose induction of the *MAL*-activator is unclear. Mal61 permease was suspected to be the maltose sensor. However, Wang et al. (2002) demonstrated that intracellular maltose is sufficient to induce *MAL* gene expression, acts via the *MAL*-activator and is independent of maltose permease protein. Whether maltose binds directly to the *MAL*-activator is unknown.

Glucose Regulation of MAL gene expression in Saccharomyces cerevisiae

MAL gene expression is maltose induced and glucose repressed. Several studies in our lab have demonstrated that glucose acts at several levels to regulate both transcriptional and post-transcriptional processes involved in maltose permease, maltase and *MAL*-activator synthesis and stability. The overall result of these multiple regulatory pathways is the rapid transition from maltose to glucose utilization. *MIG1* encodes a DNA binding transcription repressor that is active in glucose grown cells. Mig1p is negatively regulated by Snf1 kinase, which, in absence of glucose, phosphorylates Mig1p and causes it to exit the nucleus thereby releasing its target genes from repression (Carlson, 1999; Johnston, 1999).

Mig1 is a Cys_2-His_2 zinc finger DNA-binding protein, which exerts its repressive effects by recruiting a complex containing the transcription repressors Tup1p and Ssn6p (reviewed in (Gancedo, 1998). Hu et al. (1995) reported Mig1 repressor binding to sites upstream of each of the genes located at the *MAL* locus. They showed that loss of *MIG1* relieves glucose repression and increases maltase expression about 20-fold in glucose grown cells, almost to levels observed in glycerol/lactate (uninduced/non-repressed) growth conditions, whereas its over-expression results in reduced levels of maltase in both induced and uninduced conditions. They concluded that the residual glucose repression of maltase expression is due to Mig2 repressor (Hu et al., 2000).

Mig2p encodes a DNA-binding protein with homology to Mig1p (Cys_2His_2 zinc-finger-protein) that represses *SUC* and *GAL* gene expression in the presence of glucose (Lutfiyya and Johnston, 1996). Hu et al. (2000) found that in

mig1Δ and *mig1Δ mig2Δ* strains carrying a constitutive *MAL*-activator, maltose induction is still blocked in the presence of glucose. Maximal *MAL* gene expression could be observed in *mig1Δ* strains carrying a constitutive *MAL*-activator, suggesting that glucose is inhibiting maltose induction by a mechanism that is independent of Mig1, 2p. This phenomenon is referred to as Mig1 repressor-independent glucose inhibition of maltose induction.

Maltose permease is required for maltose induction because it is needed to bring maltose (inducer) into the cells (Charron et al., 1986). One possible mechanism of glucose inhibition is “inducer exclusion”. Abundant studies in our lab (Medintz et al., 1996; Medintz et al., 1998; Medintz et al., 2000) showed that maltose permease is ubiquitinated and rapidly degraded following the addition of glucose to maltose fermenting cells. Hu et al. (2000) found that Mig1-independent glucose inhibition was observed even under conditions where a low but significant maltose transport activity was expressed. They concluded that glucose inhibition of *MAL* gene induction was not due to inducer exclusion.

Hu et al. (2000) demonstrated that a constitutive *MAL*-activator that binds even in the presence of glucose is insufficient to relieve glucose inhibition. They explored the role of several known negative regulators of glucose repression pathway including hexokinase (Hxk2p), Reg1p, Gsf1p and Grr1p. Each of these negatively regulates Snf1 kinase which is required for maltose induction. Their results suggested that Snf1 kinase may play a role in reversing glucose inhibition, and indicated a novel post-transcriptional function for Snf1p in the synthesis of active, plasma membrane localized maltose permease.

Snf1 kinase (review)

SNF1, for sucrose non-fermenting, encodes a 72kDa serine/ threonine protein kinase that is homologous to the catalytic subunit of the mammalian AMP-activated protein kinase (Celenza and Carlson, 1986). AMPK is a metabolic regulator and an important target for drug development against diabetes, obesity and other diseases. It is activated by increasing AMP: ATP ratio and acts to shut off energy demanding biosynthetic processes and stimulate energy producing catabolic processes (Vincent et al., 2001). AMPK appears to be highly conserved in all eukaryotes as its homologues exist in mammal, plants and fungi. All AMPK appear to have three subunits α (Snf1p), β (Sip1,2p or Gal83p), and γ (Snf4p) (Hardie and Carling, 1997).

Glucose does not regulate *SNF1* transcription and instead controls Snf1 kinase function by inhibiting its catalytic activity. The Reg1-Glc7 phosphatase complex plays a role in Snf1p inactivation. Reg1 interacts with the catalytic domain of Snf1p under high glucose conditions and targets Glc7p to dephosphorylate the Snf1p catalytic domain thereby inactivating Snf1 kinase (Ludin et al., 1998). The carboxy-terminal regulatory domain of Snf1 kinase masks its amino-terminal catalytic domain in the presence of glucose (reviewed in (Carlson, 1999; Johnston, 1999). Upon glucose removal Snf4p, the Snf1 kinase activating subunits, binds the regulatory domain of Snf1p and activates the enzyme complex (Celenza and Carlson, 1989). Thus Snf4p and Reg1p act in opposition to control the activity of the catalytic domain of Snf1p via controlling the phosphorylation of the Snf1p regulatory domain.

Snf1p-Snf4p complex also interacts with members of the Sip family of proteins (Sip1p, Sip2p and Gal83p), which serve as bridging proteins for the protein complex and may act to recruit substrates (Jiang and Carlson, 1997). In glucose grown cells, all three proteins are localized to the cytoplasm; however, in the absence of glucose, each is differentially located. Sip1p is mainly localized to the periphery of the vacuole, Sip2p is cytoplasmic, and Gal83p is found in the nucleus (Vincent et al., 2001). The distinct regulation of Snf1 kinase activity and localization affects transcriptional control of particular genes differently, depending on whether Snf1p-regulated activators and repressors of the gene are restricted to the nucleus or shuttle between the nucleus and cytoplasm.

Activation of Snf1 kinase requires glucose starvation and any one of three upstream kinases, Sak1, Tos3 and Elm1. These Snf1p-activating kinases phosphorylate the activation loop threonine of Snf1p even in absence of the beta and gamma subunits of the Snf1 kinase complex (Elbing et al., 2006). Cells expressing only one of the three upstream kinases exhibit distinct abilities to activate Snf1p, depending on the beta subunit present in the Snf1 kinase complex and the stress imposed on the cells. Pak1p and Gal83p are the most promiscuous (McCartney et al., 2005). Once active, Snf1 kinase acts to inhibit repression by Mig1p and other transcriptional regulators, such as Adr1p and Cat8p (Treitel *et al*., 1998). Mig1p functions downstream of Snf1p in the pathway as evidenced by the finding that defects in *SUC2* and *GAL1* expression in *snf1* mutants can be suppressed by a *mig1* mutation (Johnston et al., 1994; Vallier and Carlson, 1994). Treitel et al. (1998) showed that Snf1p is required for the

phosphorylation of Mig1p *in vivo* and that the two proteins interact in two-hybrid studies and co-immunoprecipitate.

In addition to inhibition of Mig1p in absence of glucose, Snf1 kinase is required for activation of Sip3p and Sip4p, both of which have been shown to be transcriptional activators (Lesage et al., 1994; Lesage et al., 1996). Snf1 kinase has also been shown to interact with Gcn5p, the histone acetyltransferase catalytic subunit of the yeast coactivator complex and regulates transcription via chromatin remodeling (Wan-Sheng *et al*., 2001). Moreover, Snf1p directly regulates transcription by RNA polymerase II holoenzyme by interacting with Srb10p, a poorly understood negative regulatory component of the yeast mediator complex (Kuchin et al., 2000). In summary, the demonstrated mechanisms by which Snf1 kinase regulates gene expression all appear to act via the phosphorylation of transcription activators and repressors of different classes.

SNF1 protein kinase is required for maltose permease synthesis

As discussed above, the results of Hu et al. (2000) suggested a role for Snf1 protein kinase in relieving glucose inhibition of maltose induction. This conclusion was based on their observation that the glucose sensing/signaling pathway for glucose inhibition shares components of the glucose repression pathway. As noted above, *snf1Δ* strains are unable to ferment sucrose, galactose and maltose, however, *SUC* and *GAL* gene transcription can be restored in *snf1Δ* strains by disrupting *MIG1.*

In order to test whether Snf1 kinase played a similar role in *MAL* gene expression, Hu et al. (2000) deleted *mig1* in a *snf1* defective strain. They found that a *snf1Δ mig1Δ* double deletion strain carrying an inducible *MAL*-activator gene could not ferment maltose and no maltase or maltose permease expression was observed even in the presence of maltose. Introduction of a constitutive *MAL*-activator gene into this *snf1Δ mig1Δ* strain surprisingly also did not allow the strain to ferment maltose. Maltase mRNA, maltose permease mRNA, and maltase were constitutively expressed but no maltose transport activity could be detected. This suggested that inhibition of *MAL* gene induction results from a post-transcriptional block in maltose permease expression and that Snf1 kinase is required to relieve that block.

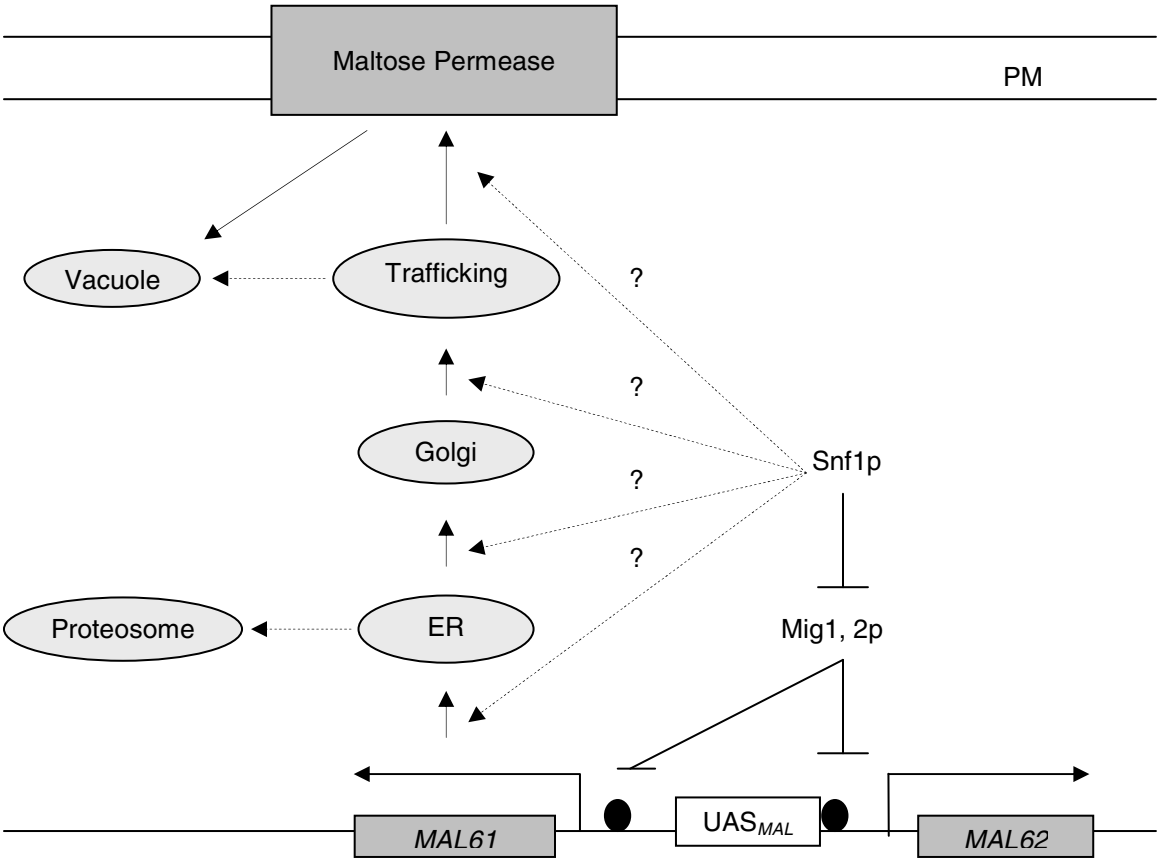
Dong and Dickson (1997) observed a similar phenomenon with the *LAC/GAL* regulon, required for lactose and galactose utilization in *Kluyveromyces lactis*. Utilization of lactose or galactose requires induction of transcription of *KlLAC4* (β-galactosidase), *KlLAC12* (lactose permease), *KlGAL1* (galactokinase), *KlGAL7 (*galactose-1-1phosphate uridylyltransferase) and *KlGAL10* (uridine diphosphoglucose-4-epimerase). *KlLAC4* and *KlLAC12* and *KlGAL1* and *KlGAL10* are each transcribed from a common bidirectional promoter. Deletion of *KlSNF1* reduces the lactose transport activity and no induction occurs. They also demonstrated that analogous to *S. cerevisiae*, *K. lactis* Mig1p regulates the *LAC/GAL* regulon in a glucose regulated manner. However, loss of *mig1* in *K. lactis* showed only a partial reversal of the *klsnf1* phenotype. Wiedemuth and Breunig (2005) demonstrated that loss of *KlSNF1*

kinase affects the lactose and galactose transporter Lac12p. Their results indicate that *LAC12* transcription was only weakly impaired and constitutive induction of *LAC/GAL* regulon was insufficient to allow full growth on lactose. They propose that Lac12 permease trafficking is impaired in *klsnf1* strain as evident by non-accumulation of Lac12-GFP fusion protein at the plasma membrane.

Figure 3 proposes possible points in maltose permease synthetic pathway that may require Snf1 kinase. Whether Snf1 kinase acts directly or indirectly is unknown. The goal of my thesis is to determine the post-transcriptional role of Snf1 kinase in maltose permease synthesis.

Figure 3. Proposed check points in maltose permease expression

Whether Snf1 kinase is directly or indirectly involved in Mal61p expression is unknown. However, possible points along the Mal61p synthetic and trafficking pathway which may require other Snf1 kinase functions are shown.



MATERIALS AND METHODS

Yeast Strains

All yeast strains used in this study are described in Table 1. Strain FY250 (*MAT**α** MAL11 MAL12 mal13 MAL31 MAL32 mal33 ura3-52 his3-Δ200 trp1-Δ63 leu2-Δ1*) is an S288C derivative. FY251 is isogenic to FY250 except for MAT**a**. Strain PS5010-1b is also isogenic to FY250 except for *snf1-Δ10* which is a deletion of almost the entire ORF and is described in Celenza *et al*., (1989). All of the above strains were a gift from Dr. Peter Sherwood and Dr. Marian Carlson of Columbia University. CMY101 is isogenic to PS5010-1b except for *mig1Δ:LEU2* which was obtained by replacing it with a functional copy of *LEU2* and is described in Hu et al., (2000). All strains carry partially functional copies of *MAL1* and *MAL3* encoding maltose permease and maltase genes. All of these strains lack a functional *MAL*-activator; therefore they are unable to ferment maltose. Introduction of a plasmid-borne copy of the *MAL*-activator gene is required for expression of the maltose permease and maltase genes.

Different players in the parallel ER associated degradation (ERAD) pathways were knocked out by PCR based one step gene replacement in CMY101 *(snf1Δ mig1Δ*) to create the deletion strains listed in Table 1. The existing sequences were replaced with either the *Kanamycin*^{R} (*G418*^{R}) or *Hygromycin B*^{R} (*HYG B*^{R}) drug resistance genes which were amplified from pFA-KanMX2 (Wach et al., 1994) or pAG32 (Goldstein and McCusker, 1999) respectively. The marker gene cassette flanked by sequence homologous to each gene to be deleted, at least 40 base pairs, was amplified by PCR using the

Table 1. Yeast Strains

Strain	Genotype	Reference
FY250	*MAT**α** MAL11 MAL12 mal13 MAL31 MAL32 mal33 ura3-52 his3-Δ200 trp1-Δ63 leu2-Δ1*	Sherwood and Carlson, 1997
FY251	*MAT**a** MAL11 MAL12 mal13 MAL31 MAL32 mal33 ura3-52 his3-Δ200 trp1-Δ63 leu2-Δ1*	Sherwood and Carlson, 1997
PS5010-1b	*snf1-Δ10* otherwise isogenic to FY250	Carlson and Celenza, 1989
CMY101	*mig1Δ::LEU2* otherwise isogenic to PS5010-1b	Hu et al. 2000
CMY102	*mig1::KAN^R* otherwise isogenic to FY250	This study
CMY103	*gal80::HYGB^R* otherwise isogenic to CMY101	This study
CMY104	*doa4::HYGB^R* otherwise isogenic to CMY101	This study
CMY105	*pep4::HYGB^R* otherwise isogenic to CMY101	This study
CMY106	*ubc6::HYGB^R* otherwise isogenic to CMY101	This study
CMY107	*ubc7::HYGB^R* otherwise isogenic to CMY101	This study
CMY108	*ubc6::KAN^R* otherwise isogenic to CMY107	This study
CMY109	*cue1::HYGB^R* otherwise isogenic to CMY101	This study
CMY110	*bsd2::HYGB^R* otherwise isogenic to CMY101	This study
CMY111	*pep12::HYGB^R* otherwise isogenic to CMY101	This study
CMY112	*eps1::KAN^R* otherwise isogenic to CMY101	This study
CMY113	*der3::HYGB^R* otherwise isogenic to CMY101	This study
CMY114	*doa10::HYGB^R* otherwise isogenic to CMY101	This study
CMY115	*rck2::KAN^R* otherwise isogenic to CMY101	This study
CMY116	*ire1::HYGB^R* otherwise isogenic to CMY101	This study
CMY117	*end3::KAN^R* otherwise isogenic to CMY101	This study

Table 2. Primers Used For One Step Gene Replacement

Primer Name	Sequence*
HygB-check	5`-TTC CTT TGC CCT CGG ACG AGT-3`
X11-Kan-check	5`-CCT CGA CAT CAT CTG CCC-3`
PEP12-A	5`-TCG GAA GAC GAA TTT TTT GGT GGT GAT AAT GAA GCC GTT TGG CAG CTG AAG CTT CGT ACG C-3`
PEP12-C	5`-CCC TCC ATC TGC TCG TAC GTT TTT GGT ACC GCA TGG CCT TCC GCA TAG GCC ACT AGT GGA TCG G-3`
PEP12-up	5`-CAG GCC TGC TAT CAC TCC AA-3`
RCK2-up	5`-CCA GTG ATT AAG AGG AAA CCT CGT CAA GAA TCC TAG ATA ATA GTA CAG C-3`
RCK2-down	5`-GAA GTG TTG ACT AAG AAA CGG GAA TCA ACA TTC ACT TGC TTA TCT GCA T-3`
X24C-MIG1D	5`-GAA GCA ACA ACA AAT TTT TAC AAC A -3`
X24L-MIG1D	5`-CAC GAG AGT TGA GTA TAG TGG AGA CGA CAT ACT ACC ATA GCC ATG CAG CTG AAG CTT CGT ACG C-3`
X24R-MIG1D	5`-TGT CTT TTG ATT TAT CTG CAC CGC CAA AAA CTT GTC AGC CTA TCA GCA TAG GCC ACT AGT GGA TCT G-3`
SC26-DOA4L	5`-CTT CGG TAG TAT AGG GCA GAT TTA AGA CTG AGT GTG CAC GCT TCC CAG CTG AAG CTT CGT-3`
SC27-DOA4R	5`-GCC ATG TTG AAC GGG AAA AAA AGT GTA TAG ACA ACG GTT TTC AGT GCA TAG GCC ACT AGT-3`
SC28-DOA4C	5`-TAT AAC CAT TAT TTA TTT GCT-3`

Table 2 (Continued)

Primer Name	Sequence*
SC29-GAL80L	5`-TCA CTG CTG GTC CTT GCC GAC CAG CGT ATA CAA TCT CGA TAG TTG GCA GCT GAA GCT TCG T-3`
SC30-GAL80R	5`-AAC GTT CGC TGC ACT GGG GGC CAA GCA CAG GGC AAG ATG CTT TTA GCA TAG GCC ACT AGT-3`
SC31-GAL80C	5`-TAT ACC CCT TTC TTC TCT CCC-3`
SC34-DER3L	5`-ATC TAT CAA TTG CAA TTT GTA AGA GAA GGG GAG AAA GAC AAA ATC AGC TGA AGC TTC GTA CGC-3`
SC35-DER3R	5`-ATA AAA CAT GCA ATC TAG ATA TGC TGG ATA AAT TTA TCT GGT ATG GCA TAG GCC ACT AGT GGA T-3`
SC36-DER3C	5`-CCC CCC TAC CAT TTC TAA TAA-3`
SC45-UBC7C	5`-GGC GTT TAG CGT ACG AAG GAG-3`
SC46-UBC7L	5`-CCG CTC AGA AAC GTC TCC TCA AGG AGC TTC AAC AGT TAA TCA GCT GAA GCT TCG T-3`
SC47-UBC7R	5`-CTA ATG ATT TCA AAA TGG ATA ACT TTA CCT GTC TCT CAA AGC ATA GGC CAC TAG T-3`
SC48-UBC6C	5`-GAC TAC CAT CGC ATA TCG CAG-3`
SC49-UBC6L	5`-ACG AAA GAG TAC AAG TTG ATG GTG GAA AAC CCT CCA CCA TCA GCT GAA GCT TCG T-3`
SC50-UBC6R	5`-CCA ATA TAA ACC ATT GAA GAA CTA TCA TTA GGT TCT TTG CGC ATA GGC CAC TAG T-3`
SC70-EPS1L	5`-ATG AAA ATG AAT CTG AAA AGG CTC GTA GTT ACC TTC TTC TCA GCT GAA GCT TCG T-3`
SC71-ESP1R	5`-AAC TAC TTA AGC GTT TAA TCT TGA TTT TTT TTT TTC TCC AGC ATA GGC CAC TAG T-3`

Table 2 (Continued)

primer Name	Sequence*
SC72-EPS1C	5`-GAT CAT AGC ACT AAG AAG GCG-3`
SC73-CUE1L	5`-ATG GAG GAT TCG AGA TTG CTT ATC ACT TTG ATT CTT GTG TCA GCT GAA GCT TCG T-3`
SC74-CUE1R	5`-TGC GTG TTC CG ACA AGC ACT TAA GCG TTC AAG TCA GCA AGC ATA GGC CAC TAG T-3`
SC75-CUE1C	5`-GGG TAG CTG TCG CGC CAT TTT-3`
SC76-DOA10L	5`-ATG GAT GTT GAT TCT GAC GTT AAT GTC TCC AGG TTA AGA GCA GCT GAA GCT TCG T-3`
SC77-DOA10R	5`-TCT GGT AAA TTT TCT AAA GCT CTA CCC TTA GTG TAA ACC TGC ATA GGC CAC TAG T-3`
SC78-DOA10C	5`-CAT TAG AGA CGG AAA ACT GGT-3`
SC87-BSD2C	5`-CCG GAG ATG TAA ATA AC GTG-3`
SC88-BSD2L	5`-AGG TTC ATC TAC TGA TGG CAT AAA TGT CGG AAACGC AGG ACA GCT GAA GCT TCG T-3`
SC89-BSD2R	5`-TAT ACT CTT CAG GGT GAT AAA TAG CCC AAG AAA TGC CAC AGC ATA GGC CAC TAG T-3`
SC93-PEP4C	5`-GAG AAG CCT ACC ACG TAA GGG-3`
SC94-PEP4L	5`-GTG ACC TAG TAT TTA ATC CAA ATA AAA TTC AAA CAA AAA CCA AAA CTA ACC AGC TGA AGC TTC GT-3`
SC95-PEP4R	5`-ACC AAC CGC ATT GTT GCC CAA ATC GTA AAT AGA ATA GTA TTT ACG CAA GAG CAT AGG CCA CTA GT-3`

Table 2 (Continued)

primer Name	Sequence*
SC135-IRE1C	5`-TGG GCT TTT AGG GAC AGT TCT-3`
SC136-IRE1L	5`-ATG CGT CTA CTT CGA AGA AAC ATG TTA GTA TTG ACA CTG CTC GTT CAG CTG GAA GCT TCG T-3`
SC137-IRE1R	5`-ACA AAA TTC ACG TAA AAT TTG ATC GTC ACT TAA ATT TTC GCA TAG GCC ACT AGT-3`
SC142-END3C	5`-CTC GCT GCT TGT CCG TCT CGC-3`
SC143-END3L	5`-ATG CCC AAG TTG GAA CAA TTT GAA ATA AAA AAA TAC TGG CCA GCT GAA GCT TCG T-3`
SC144-END3R	5`-ATG CTT GCA ATT CGT GTC TCT TAT TGT TCA AGT AAT TCT CGC ATA GGC CAC TAG T-3`

L – Primer at 5` end of the sequence.
R – Primer at 3` end of the sequence.
C – Check primer at 5` end of the L primer.
* – The underlined sequence was used to amplify the *KAN*^{R} and *HYGB*^{R} cassettes from pKanMX4 and pAG32 respectively.

appropriate plasmid as a template. The 5` and 3` primers used to amplify the cassette and those that were later used to confirm the correct insertion are listed in Table 2. The resulting fragment, which is homologous to the target gene at both the 5`and 3` ends, was transformed into the *snf1Δ mig1Δ* strain CMY101, and the transformants were allowed to recover in YPD over night before they were selected for growth on YPD plates containing either Geneticin (200µg/ml) or Hygromycin (200-400µg/ml). Gene disruption in potential candidates was confirmed by PCR. Table 3 summarizes the gene deletions, the drug resistance gene used, the plasmid they were amplified from and the primers used to amplify them.

Yeast Genomic Library

Yeast genomic library used for this study is a high copy YEp24 library obtained from Dr. Marian Carlson's lab and is described in Botstein and Carlson (1982). Genomic DNA from *S. cerevisiae* strain S288C was partially digested using *Sau*3A restriction enzyme and DNA fragments of approximately 10kb were isolated by sucrose gradient. These were subcloned into the *Bam*HI site of YEp24, a 2-micron yeast shuttle vector. YEp24 plasmid replicates as high-copy extra-chromosomal plasmid and is present in approximately 50 copies per cell.

Plasmid Construction

Table 4 lists all plasmids, including those previously described and those constructed during the course of this study.

Table 3- Gene disruption described

Gene Deleted	Primers used	Replaced with	Amplified from
MIG1	X24L, X24R	*Kanamycin*^{R}	pFAkan-MX2
PEP12	PEP12A, PEP12C	*Hygromycin B*^{R}	pAG32
DOA4	SC26, SC27	*Hygromycin B*^{R}	pAG32
DOA10	SC76, SC77	*Hygromycin B*^{R}	pAG32
PEP4	SC94, SC95	*Hygromycin B*^{R}	pAG32
DER3	SC34, SC35	*Hygromycin B*^{R}	pAG32
UBC6	SC49, SC50	*Hygromycin B*^{R}	pAG32
UBC6	SC49, SC50	*Kanamycin*^{R}	pFAkan-MX2
UBC7	SC46, SC47	*Hygromycin B*^{R}	pAG32
GAL80	SC29, SC30	*Hygromycin B*^{R}	pAG32
CUE1	SC73, SC74	*Hygromycin B*^{R}	pAG32
BSD2	SC82, SC89	*Hygromycin B*^{R}	pAG32
EPS1	SC70, SC71	*Kanamycin*^{R}	pFAkan-MX2
RCK2	RCK2up, RCK2down	*Kanamycin*^{R}	pFAkan-MX2
IRE1	SC136, SC137	*Hygromycin B*^{R}	pAG32
END3	SC143, SC144	*Kanamycin*^{R}	pFAkan-MX2

Table 4. Plasmids

Plasmid	Reference
pUN30/MAL63/43-c	(Gibson et al., 1997)
pUN30/MAL61/HA	(Medintz et al., 2000)
p413/GPD-MAL63/43-c	(Bali et al., 2003)
pCE9 (YEp24/SNF1)	(Celenza and Carlson, 1989)
pRS315/MAL61/HA	(Medintz et al., 1996)
pUN30/SNF1	This study
YEp24/RCK2	This study
YEp24/YEF3	This study
YEp24/GAL11	This study
YEp24/GSH2	This study
YEp24/TRL1	This study
p424/TRL1	This study
YEp24/EXO70	This study
p424/EXO70	This study
YEp24/YJL086C	This study
YEp24/YJL084C	This study
p423/GPD-YJL084C	This study
p424/RGD2	This study
YEp24/RGD2	This study
YEp24/EMP47	This study
p423/GPD-EMP47	This study

Table 4 (Continued)

Plasmid	Reference
YEp24/ALR2	This study
YEp24/YFL049W	This study
p424/YFL049W	This study
pUN70/MAL61/HA/His6-GFP	(Gadura and Michels, 2006)
p423/GPD -MAL61/HA/His6	This study
p424/GPD -MAL61/HA/His6	This study
p425/GPD -MAL61/HA/His6	This study
p426/GPD -MAL61/HA/His6	This study
p414/GPD -HXT1/HA	This study
p426/GPD -HXT1/HA	This study
p414/GPD -GAL2/HA	This study
p426/GPD -GAL2/HA	This study
YCp355/ I_{MAL61} (-862→+1)	(Hu et al., 1995)
YCp355/MAL61/HA-Lac Z (47)	This study
YCp355/MAL61/HA-Lac Z (99)	This study
YCp355/MAL61/HA-Lac Z (122)	This study
YCP355/MAL61/HA-Lac Z (294)	This study
YCp355/MAL61/HA-Lac Z (364)	This study
p426/GPD M1-293/G264-574/HA (SCMG)	This study
p426/GPD G1-263/M294-614/HA (SCGM)	This study
p426/GPD M1-99/G67-574/HA (SCMG99)	This study

Table 4 (Continued)

Plasmid	Reference
p426/GPD G1-66/M100-614/HA (SCGM66)	This study
p426/GPD M1-178/G151-574/HA (SCMG178)	This study
p426/GPD G1-150/M179-614/HA (SCGM149)	This study
p426/GPD-MAL61/HA (G-TMD1) (SCMG-1st TMD swap)	This study

Genomic DNA fragments containing single genes from the multi-copy suppressor plasmids, MCS31, MCS54, MCS56 and MCS59 were amplified by PCR using the suppressor plasmids as the template. Primer pairs complimentary to each gene were designed with the restriction digest sites, required for insertion into the recipient vector, at the termini. YEp24 is a 2μ plasmid with several unique restriction enzyme digest sites available for subcloning in its multiple cloning site. Any one or two of these sites were used to sub-clone the PCR-amplified genomic fragments into the plasmid. The multicopy vectors p424 and p423, from the Mumberg series of plasmids designed to construct GPD fusions genes, were also used to subclone genes from the MCS31 and MCS56 plasmids (Mumberg et al., 1995). All genes, unless stated otherwise, carried at least 350 bp of upstream sequence. Table 5 describes the primers, restriction digest sites, and vectors used to sub clone individual genes on each of the multi copy suppressor. Detailed sequence of each of the primers used can be found in Table 6.

To create pUN30/SNF1, pCE9 (YEp24/SNF1) provided by Dr. Carlson was digested with *Eco*RI and *Bam*HI. A 3.2 kb fragment containing the promoter and the entire *SNF1* ORF was subcloned into the *Eco*RI-*Bam*HI sites of pUN30, a CEN vector (Elledge and Davis, 1988).

MAL61-LacZ fusion genes were constructed by amplifying the entire *MAL61-62* intergenic region containing the promoter and various lengths of *MAL61* gene (to codons 47, 99, 122, 294 and 364) and inserting the amplified fragments in frame into YCp355. YCp355 is a yeast CEN vector constructed by

Table 5- Gene Subcloning described

Plasmid	Vector	Primers†	Restriction Sites
pRCK2	YEp24	A19, A20	*BamHI*, *SalI*
pYEF3	YEp24	A17, A18	*BamHI*, *Sph*I
pGAL11	YEp24	A21, A22	*BamHI*, *SalI*
pGSH2	YEp24	A23, A24	*BamHI*, *SalI*
pTRL1	YEp24	A7, A8	*BamHI*, *SalI*
pTRL1	p424	A8, A27	*BamHI*, *SalI*
pEXO70	YEp24	A5, A6	*BamHI*, *SalI*
pEXO70	p424	A28, A29	*BamHI*, *SalI*
pYJL086C	YEp24	A1, A2	*BamHI*, *SalI*
pYJL084C	YEp24	A3, A4	*Eag*I, *SalI*
pGPD/YJL084C	p423	SC100, SC117	*Spe*I, *Xho*I
pALR2	YEp24	A9, A10	*Eag*I, *SalI*
pYFL049W	YEp24	A11, A12	*Eag*I, *SalI*
pYFL049W	p424	A26, A12	*SalI*
pEMP47	YEp24	A13, A14	*BamHI*, *Nhe*I
pGPD/EMP47	p423	SC102, SC118	*Spe*I, *Cla*I
pRGD2	YEp24	A15, A16	*BamHI*, *SalI*
pRGD2	p424	A15, A16	*BamHI*, *SalI*
pEMP47-RGD2	YEp24	A14, A16	*BamHI*

† - All primer sequences are listed in Table 6

Table 6. Primers Used to Subclone Multicopy Suppressor Genes

Primer	PrimerName	Sequence*
A1	*Sal*I YJL086C	5`-GGC GTC GAC GGA TGT AGC TGC AAT T-3`
A2	YJL086C *Bam*HI	5`-GGC GGA TCC GCT CTG CCT CTA CCG G-3`
A3	YJL084C *Eag*I A	5`-GCG CGG CCG CAC TGT TTC ATT CAT CAC CCA-3`
A4	YJL084C *Sal*I B	5`-CCC GTC GAC TAC CCC TGA CGA ACT AAC TAC-3`
A5	*Bam*HI EXO70 R	5`-GCG GGA TCC GGC CAG CTT TTT CAA GGG GAT-3`
A6	EXO70 *Sal*I L	5`-GGC GTC GAC CAA CCC CAC AAC GCG AGG AAA-3`
A7	*Sal*I TRL1 R	5`-GGC GTC GAC GGA ATG CCA TGC ACT TGA GGA-3`
A8	YEp24 A *Bam*HI	5`-CCC GGA TCC CAT GGC GAC CAC ACC CGT CCT-3`
A9	ALR2 *Eag*I B	5`-GCG CGG CCG CTG AAG TGT GTC AGA ATA TGA-3`
A10	ALR2 *Sal*I A	5`-CCC GTC GAC CAT TCA TTT GAC AGA GGC CCT-3`
A11	YFL049W *Eag*I	5`-GCG CGG CCG CGA CGA AAT TGC CAG AAA AC-3`
A12	*Sal*I YFL049W	5`-CCC GTC GAC GCA CGT AGG AAG AAT AAG CGA-3`
A13	*Bam*HI EMP47 C	5`- CCC GGA TCC GAA TGA ACA AGT CGC ATT GCT-3`

Table 6 (Continued)

Primer	PrimerName	Sequence*
A14	*Nhe*I EMP47 D	5`-CCG CTA GCG CTA ATC CAG TGG TGG CGC G-3`
A15	†YFL047W *Sal*I	5`-CCC GTC GAC GCA TCT GAA GTG TCA CCC AAT-3`
A16	†*Bam*HI YFL047W	5`-CCC GGA TCC CTT GAA ACA GCT GCC TAC AGT-3`
A17	YEF3 *Bam*HI	5`-CCC GGA TCC ACT CGC AAA AGG GCC CAA CCA-3`
A18	YEF3 *Sph*I	5`-CCC GCA TGC GT CTG ACT AAT GGA ACG CTT-3`
A19	RCK2 *Sal*I	5`-CCC ATA GGG TAA CGA CCG GGT AAA -3`
A20	RCK2 *Bam*HI	5`-CCC GGA TCC GAT TAA GAG GAA ACC TCG TCA-3`
A21	GAL11 *Bam*HI	5`- CCC GGA TCC TCC ACG GAT GGT GCA GAA GT-3`
A22	GAL11 *Sal*I	5`-CCC GTC GAC AAC CCG GCC AAA AAA GCG TA-3`
A23	*Bam*HI GSH2	5`-CCC GGA TCC GGA CAA GTG CTA CTT GAA CA-3`
A24	*Sal*I GSH2	5`-CCC GTC GAC TGT AGG ACC AAT CTT GGC AA-3`
A25	ALR2 SalI B	5`-CCC GTC GAC GCC GAC CTA CCA TAG CGG TCA-3`
A26	*Bam*HI YFL049W	5`-CCC GGA TCC GAT GGT CCT TCA GGT CGA CGAA-3`

Table 6 (Continued)

Primer	PrimerName	Sequence*
A27	ASL1 TRL1C	5`-CCC GTC GAC AGC GGG CAT TTC TAT AAA TGC-3`
A28	EXO70A *Sal*I	5`-GGC GTC GAC CTA GGC ATC GCT TCT TCG TAT-3`
A29	EXO70B *Bam*HI	5`-GGC GGA TCC CTG GCG TCC AGA GCA AAC ATA-3`
SC100	*Spe*I YJL084C	5`-CCC ACT AGT ATG CCC ATG GAC CAA TCT ATC-3`
SC102	*Spe*I EMP47	5`-CCC ACT AGT ATG ATG ATG TTA ATT ACT ATG-3`
SC117	*Xho*I YJL084C	5`-TTT CTC GAG CTA AAG GGT ACT CTC ATT TAT-3`
SC118	*Cla*I EMP47	5`-TTT ATC GAT TCA TAG CAG TTT GGT CTT TAT-3`

† - An alias for *YFL047W* is *RGD2*.
* - The restriction enzyme sites are underlined.

Table 7. Primers Used For *MAL61-Lac Z* Fusions

Name	Sequence
*SC22-LAC Z-check	5`-AAA GGG GGA TGT GCT GCA AGG-3`
SC17-*Bam*HI MAL61	5`-CCG GAT CCG TTA CGC TTG ACT GAT GTA CA-3`
SC18-*Pst*I MAL61	5`-GGC TGC AGA GCT TTT GGA TAT GTC TTC AA-3`
SC20-MAL61 (1)-*Pst*I	5`-CCC CTG CAG AAG ATC AAA ATC ACT TTT CTT-3`
SC21-MAL61 (2)-*Pst*I	5`-CCC CTG CAG CTC TGG TGC CAA AAA AAT ACC-3`
SC23-MAL61 (3)-*Pst*I	5`-CCC CTG CAG TCT CGT TCT TCT CCT GTT AAT-3`
SC51-*Pst*I (7) MAL61	5`-CTG CAG AGC TCC TAG AAT GGC TGT GTC-3`

The restriction digest sites are underlined.
*- Complimentary to *Lac Z* sequence, rest of the primers have sequence complimentary to *MAL61* alone.

introducing the CEN sequence from pRS315 into vector YIp355 at the *Eco*RI site (Hu et al., 1995). YCp355 has several unique restriction sites in its multiple cloning site including *Bam*HI, *Eco*RI, *Hind*III, *Pst*I, *Sal*I, and *Sma*I located upstream of the *LacZ* gene. The 5 ` primer complimentary to the *MAL61* promoter sequence has *Bam*HI site on its terminus, while all of the 3` primers have downstream *Pst*I sites. The primer sequences are listed in Table 7. All of the amplified fragments contain the *MAL61* translation initiation sequence and AUG. The constructs were screened by PCR by using a primer complimentary to *LacZ* (SC22) andanother primer complimentary to *MAL61* sequence. The sequence of the fusion genes was further confirmed by sequencing.

To construct constitutively expressed *HXT1*, *GAL2* and *MAL61*, the multicopy plasmids from the Mumberg series (Mumberg et al., 1995) were used to fuse these to the promoter of the *TDH3* gene, referred to as GPD, which allows for high constitutive expression. Any one of the four vectors from the GPD multicopy series, p423, p424, p425 and p426, were used which carry one of the selection marker genes (*URA3*, *TRP1*, *LEU2*, *HIS3*), and the *TDH3* promoter. *GAL2*, *HXT1* and *MAL61* were PCR amplified from p121/ADH1-GAL2, pBF307/ADH1-HXT1 or pRS315/MAL61/HA respectively. The 5` primers for *GAL2* and *HXT1* included the sequence encoding the HA tag along with 21 bp of sequence homologous to either of the two genes. Each primer pair also included *Xho*I and *Cla*I restriction sites flanking the gene sequences which are required for insertion into the vector. All primers used for gene fusions with the GPD

Table 8. Primers Used To Construct Fusions with the GPD Promoter

Primer	Sequence
*†SC2-*Bam*HI His HA	5`-CCC GGA TCC ATG CAT CAT CAT CAT CAT CAT **TAC CCA TAC GAT**-3`
SC4-3` MAL61 *Sma*I	5`-CGC CCG GGT AAC GCC GAT CT ACCT AC-3`
†SC32-HA GAL2L *Eco*RI	5`-CCC GAA TTC **ATG TAC CCA TAC GAT GTT CCA GAT TAC GCT AGC TTG GGT GGT CCT** GCA GTT GAG GAG AAC AAT ATG-3`
SC33-GAL2R *Cla*I	5`-CCC ATC GAT TTA TTC TAG CAT GGC CTT GTA-3`
†SC61-*Cla*I HA	5`-CCC ATC GAT **ATG TAC CCA TAC GAT GTT CCA**-3`
SC62-*Xho*I MAL61	5`-CCC CTC GAG TCA TTT GTT CAC AAC AGA TGG-3`
SC63-*Xho*I GAL2	5`-CGC CTC GAG TTA TTC TAG CAT GGC CTT GTA-3`
†SC64-*Cla*I HA HXT1	5`-CCC ATC GAT **ATG TAC CCA TAC GAT GTT CCA GAT TAC GCT AGC TTG GGT GGT CCT** AAT TCA ACT CCC GAT CTA ATA-3`
SC65-*Xho*I HXT1	5`-GGG CTC GAG TTA TTT CCT GCT AAA CAA ACT-3`
SC100-*Spe*I YJL084C	5`-CCC ACT AGT ATG CCC ATG GAC CAA TCT ATC-3`
SC102-*Spe*I EMP47	5`-CCC ACT AGT ATG ATG ATG TTA ATT ACT ATG-3`
SC117-*Xho*I YJL084C	5`-TTT CTC CAG CTA AAG GGT ACT CTC ATT TAT-3`

Table 8 (Continued)

Primer	Sequence
C118-*Cla*I EMP47	5`-TTT ATC GAT TCA TAG CAG TTT GGT CTT TAT-3`
SC126-*Cla*I MAL61	5`-TTT ATC GAT TAA CGC CGA TCT ACC TAC TGG-3`

† - HA sequence is in bold face.
* - 6 His sequence is highlighted and underlined.
The restriction digest sites are underlined.

promoter are listed in Table 8. The constructions were confirmed by PCR, restriction digest and Western blot analysis.

Primers used to construct hybrids between Mal61 maltose permease and Gal2 galactose permease are described in Table 9. pRS315/MAL61/HA and p414/GPD-GAL2/HA were used to amplify various portions of *MAL61* and *GAL2*, respectively. Two rounds of PCR were required to construct the hybrids and accordingly two sets of primer pairs were designed. The overall scheme is diagramed in Figure 4, which shows the construction of A-B hybrid gene. In the first round of PCR, the desired region of each gene is amplified using one primer at the end of the gene (either 3` or 5`) and the second primer at the junction site. The junction site primer consists of sequence homologous to both genes and taken from both sides of the desired junction site. The product of these two amplifications are mixed and allowed to anneal, which occurs in the region of the junction site primer. The 5` ends of hybrids are used as primers for the first extension reaction thereby creating a full-length hybrid gene. The second round of PCR uses only primers from the two ends of the gene and amplifies the full-length gene, which is then inserted into plasmid p426 GPD (Mumberg *et al.*, 1995). p426/GPD M1-99/G67-574/HA was used as template to amplify the *MAL61-GAL2* hybrid portion of p426/GPD-MAL61/HA (G-TMD1). The primers used for different hybrid constructs are listed in Table 9 and Table 10 lists the primer pairs used for each hybrid construct and the junction sites.

Maltose Transport Assay

Figure 4. The scheme used to construct *MAL61* and *GAL2* hybrids

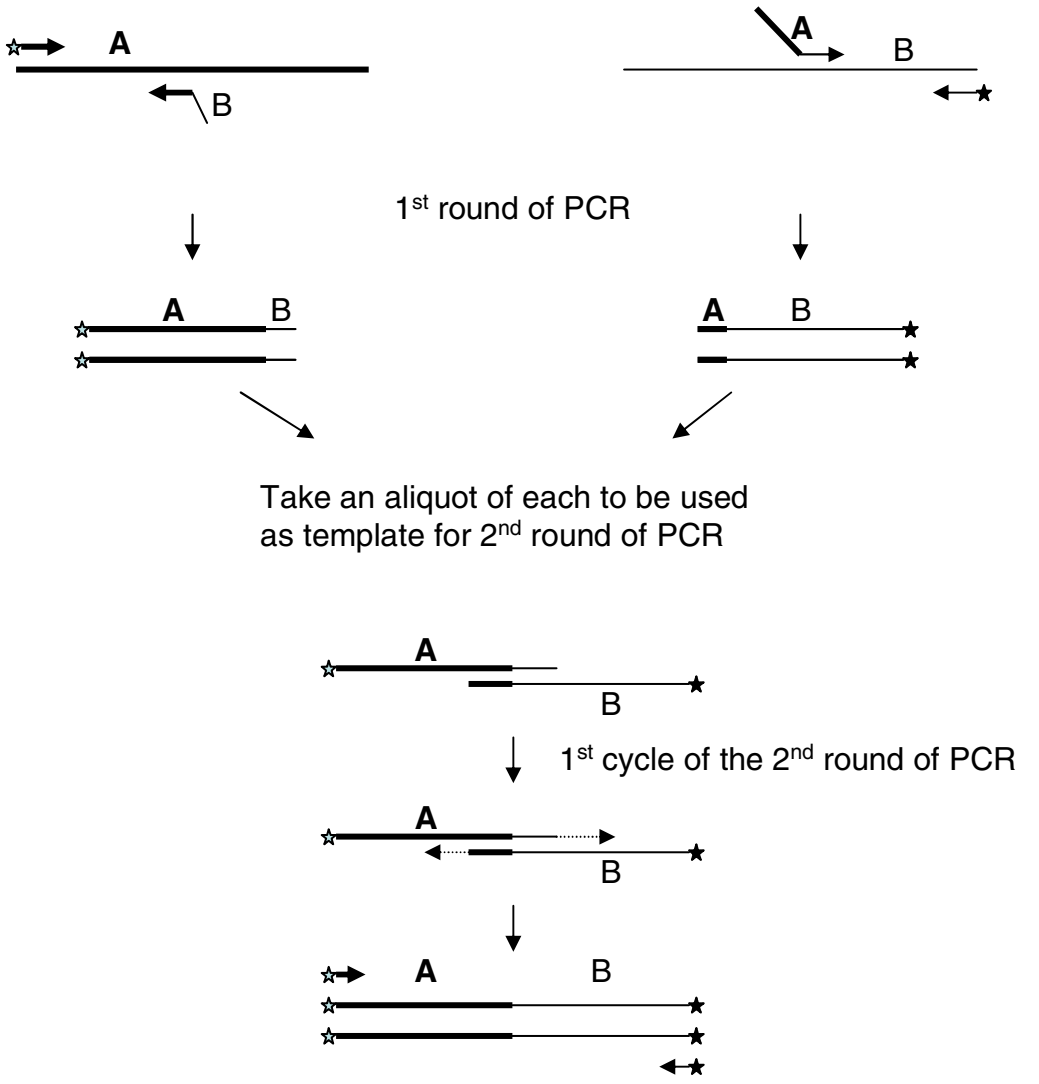


Table 9. Primers Used for *GAL2* and *MAL61* Hybrid Construction

Primer	Sequence
*SC57-MALGAL-1	5`-ATT CAC CTC ACA TAA ATA ACG TGG GGA TTC **TGG TGC CAA AAA AAT ACC TAC CGC CCA A AG G**-3`
*SC58-MALGAL-2	5`-**CCT TTG GCG GTA GGT ATT TTT TTG GCA CCA** GAA CCA CGT TAT TTA TGT GAG GTG AAT-3`
*SC59-GALMAL-1	5`-TTT ATG ATT GGC GCT TTG ACG TTA GTT CCT **GAG TCT CCA TGG TGG CTG GTT AAA AAA GGA**-3`
*SC60-GALMAL-2	5`-**TCC TTT TTT AAC CAG CCA CCA TGG AGA CTC** AGG AAC TAA CGT CAA AGC GCC AAT CAT AAA-3`
†SC61-*Cla*I.HA	5`-CCC ATC GAT ATG TAC CCA TAC GAT GTT CCA-3`
†SC62-*Xho*I.MAL61	5`-CCC CTC GAG TCA TTT GTT CAC AAC AGA TGG-3`
†SC63-*Xho*I.GAL2	5`-CGC CTC GAG TTA TTC TAG CAT GGC CTT GTA-3`
SC127-SCMG99	5`-ACA CAA ACA AAG CAA GGA AAC GGT AAC ATA TTC **AGC TTT TGG ATA TGT CTT CAA**-3`
SC128-SCMG99	5`-**CTC ATG ACA GCT TTG AAG ACA TAT CCA AAA GCT** GAA TAT GTT ACC GTT TCC TTG-3`
SC129-SCMG178	5`-AAC TAT ATA AAC CGA GAC GAC AAT CGA AAG ACC **ACG GTT GCC CAT GTA ATC TAC**-3`

Table 9 (continued)

Primer	Sequence
SC130-SCMG178	5`-**GGG CCT TCT GTA GAT TAC ATG GGC AAC CGT** GGT CTT TCG ATT GTC GTC TCG-3`
SC131-SCGM66	5`-**TGT TGT GGA AAC TAA TAG TGA CCA AGC AGC** AGA CAT GGG CTT CTT GGG TAT-3`
SC132-SCGM66	5`-CCT ATA GAG ATA CCC AAG AAG CCC ATG TCT **GCT GCT TGG TCA CTA TTA GTT**-3`
SC133-SCGM149	5`-**CGC TAA AAA GAA CAA CGC CAT GAT CAG AGT GTA** CTT TTT ACG GCC ATA CAT ATC-3`
SC134-SCGM149	5`-AAA GGT GGA GAT ATG TAT GGC CGT AAA AAG **TAC ACT CTG ATC ATG GCG TTG**-3`
SC140-SCMG 1st TMD	5`-CCA GCC AAA CAT GAA GCC GCC-3`
SC141-SCMG 1st TMD	5`-GGC GGC TTC ATG TTT GGC TGG **GGA GCT TTC TAT GCC CTG CCT**-3`

* - For hybrid primer sequences, *MAL61* sequence is in bold face and underlined, rest of the sequence belongs to *GAL2.*

† -The restriction digest sites are underlined

Table 10- *MAL61* and *GAL2* Fusions Described

Hybrid Sequence	*MAL61* Derived Sequence	*GAL2* Derived Location	Junction Site	*MAL61* Primer Pair	*GAL2* Primer Pair
†SCMG	1-293 codons	264-574 codons	3`end of 6th TMD	SC61, SC57	SC63, SC58
†SCMG99	1-99 codons	67-574 codons	5` end of 1st TMD	SC 61, SC127	SC63, SC128
†SCMG178	1-178 codons	150-574 codons	5` end of 3rd TMD	SC61, SC129	SC63, SC130
SCMG (G-TMD1)	1-99 codons and	67-87 codons	5`-3` of 1st TMD	SC61, SC140	
	122-574 codons			SC62, SC141	
*SCGM	294-614 codons	1-263 codons	3`end of 6th TMD	SC59, SC62	SC60, SC61
*SCGM66	100-614 codons	1-66 codons	5` end of 1st TMD	SC62, SC132	SC61, SC131
*SCGM149	179-614 codons	1-149 codons	5` end of 3rd TMD	SC62, SC134	SC61, SC133

† - SC61 and SC63 are the primer pair for the 2nd PCR reaction.
* - SC61 and SC62 are the primer pairs for the 2nd PCR reaction.

Yeast strains were grown to mid log phase in YNB minimal medium containing the appropriate amino acids plus 0.1% glucose. Cells were then harvested, washed with sterile water and resuspended in the same minimal medium plus 2% maltose as the carbon source for six hours before the maltose transport activity was assayed. Alternatively, cells were grown to mid-log phase in YNB minimal medium plus 0.1% glucose without maltose induction. All assays were done in duplicates on at least three independent transformants. Maltose transport is measured as the rate of uptake of [^{14}C]-maltose (Amersham Cat #CFB-182)as previously described (Cheng and Michels, 1991). Maltose transport activity is expressed as nanomoles of radioactive maltose transported per milligram dry weight of cells per minute.

Galactose Transport Assay

Mid log cells grown in uracil drop out selective medium complete with 0.1% glucose were harvested, washed with 0.1M TTA pH 4.2 and resuspended in 330µl of the same buffer. 80µl samples were taken and incubated with ^{14}C-galactose (Amersham Cat #CFB-132) for 10 seconds at room temperature. Cells were collected by vaccum filtration, washed three times with ice-cold water, and subjected to scintillation counting. All assays were done in duplicates on at least three independent transformants. Galactose transport activity is expressed as nanomoles of radioactive galactose transported per milligram dry weight of cells per minute.

Mal61p Turn Over Assay

Cells were grown to early log phase (0.3-0.5 OD_{600}) overnight at 30° C in selective medium containing 0.1% glucose. At time zero cyclohexamide (50µg / ml) was added to inhibit protein synthesis. OD_{600} was determined and a sample was removed at time zero and every hour for three hours thereafter. Samples were harvested by filtration and frozen at -80° C until used for western blot analysis. Growth dilution was determined as OD_{600} at time zero divided by OD_{600} a time x.

Western Blot Analysis

Cells were grown to early log phase (0.3-0.5 OD_{600}) overnight in the appropriate drop out medium containing 0.1% glucose at 30° C. In some cases cultures were diluted in the morning and returned to the incubator for another three hours before cell collection. Fifteen O.D. units of mid-log phase cells were harvested by filtration through nitrocellulose filters (0.45µm), washed with 50mM KPO_4 plus 0.1% sodium, azide pH 7.4 and frozen at -80° C in Falcon tubes for at least 20 minutes before whole cell protein extracts were prepared. Cells from the filter were recovered by resuspending in 1mL of 50mM HEPES buffer, pH 7.5. The following protease inhibitors were added to the HEPES buffer just before using: manufacturer recommended amount of Sigma protease inhibitor cocktail (Product # P8215) containing AEBSF, pepstatinA, E-64 and 1, 10 phenanthroline. Cells were harvested by centrifugation and resuspended in 250 µL of extraction buffer containing 40mM TrisCl pH6.8, 8M Urea, 0.1M EDTA, 1% β-mercaptoethanol, and 5% SDS complemented with the protease inhibitor cocktail

as described above . Cells were broken open by vortexing with glass beads at 4° C for 15 minutes, followed by solubilization at 37° C for 15 mins. To this suspension, another 40µL of extraction buffer were added and tubes were again vortexed for 2 mins at 4° C before cell debris was removed by centrifugation at top speed in a microfuge for 5 mins. The supernatant was transferred to an eppendorf tube and boiled for 3 minutes before being frozen at -80° C or used for Western blot analysis. Protein levels were determined as described in Medintz et al. (1996).

Equal amounts of protein (or in some cases equal volumes of extract) were loaded and size separated using 10% SDS PAGE gels. Proteins were transferred to PVDF membrane using transfer buffer (0.303% Tris, 1.44% glycine and 20% methanol). Membranes were blocked with 10% milk for an hour before they were probed with antibody. Mal61p/HA was detected using Anti-HA antibody (Boehringer Cat. # 11583816001) and Phosphoglycerate kinase (PGK) was probed with anti-PGK antibody (Molecular Probes Cat. # A6457). Protein was visualized using an Amersham Vistra ECF kit (Cat. # RPN5781) in which secondary antibody is conjugated to fluorescent dye. The signal was visualized using a Molecular Dynamic Storm 860 and quantified using software provided by the manufacturer. PGK levels were used as a control to adjust for loading variations.

Fluorescence Microscopy

Cells were transformed with pUN70/MAL61/HA-GFP and p413/GPD

MAL63/43-c. Cultures were grown in selective medium lacking histidine and tryptophan with 0.1% glucose to mid-log phase. Samples were collected and viewed with Meridian/Olympus IMT-2 confocal microscope using a 100X oil, NA 1.40 lens and a FITC filter o by phase optics.

β-galactosidase Assay

β-galactosidase activity was assayed according to the protocols of Hu et al. (1999) using crude cell extracts. Activity is expressed as the nanomoles of O-nitrophenyl-β-D-galactopyranoside (ONPG) hydrolyzed per milligram of total protein per minute.

β-gal plate tests were done as previously described (Wang and Michels, 2004). Individual colonies are patched onto appropriate plates containing 2% maltose as the sole carbon source and are allowed to grow for two days before the test. An agarose solution containing substrate X-gal is poured over the patched colonies and color is allowed to develop for 3-6 hours. The overlay contains 0.5% agarose in 0.5 M $NaPO_4$, pH 7, 0.1% SDS, 2% DMF, and 0.05% X-gal.

CHAPTER 1

SNF1 Is Required At a Post-Transcriptional Level For The Expression Of Maltose Permease Protein

INTRODUCTION

In *Saccharomyces cerevisiae*, *MAL* gene expression is regulated by glucose via multiple mechanisms that include both Mig1 repressor-dependent and Mig1 repressor-independent mechanisms. Mig1p binding sites are present upstream of *MAL61*, *MAL62*, the *MAL*-structural genes, as well as in the upstream region of *MAL63*, the *MAL*-activator gene (Hu et al., 1995). Hu et al. (2000) showed that deletion of *MIG1* relieved glucose repression and increased maltase expression about 25-fold. However, *MAL* induction is nonetheless fully blocked in a *mig1Δ* strain in the presence of glucose. They referred to this phenomenon as *MIG1*-independent glucose inhibition and upon further investigation uncovered a novel role for Snf1 protein kinase in *MAL* gene expression.

Glucose is the preferred carbon source for *Saccharomyces cerevisiae*. Snf1 kinase is required for expression of genes needed to metabolize alternative carbon sources including maltose, sucrose, galactose, and non-fermentable carbon sources such as ethanol. Snf1 kinase is active only in the absence of glucose and Mig1 repressor is one target of Snf1 kinase (Carlson, 1999; Johnston, 1999). Snf1 kinase negatively regulates Mig1 repressor and thereby releases genes required for utilization of alternative carbon source from Mig1-mediated repression of transcription. Thus *SNF1* mutant strains are unable to ferment sucrose, galactose and maltose. Sucrose fermentation can be restored in a *snf1Δ* strain by loss of Mig1 repressor. The sole role for Snf1p kinase in sucrose utilization is to relieve the Mig1p repression of *SUC* gene transcription.

In contrast to sucrose utilization, (Hu et al., 2000) suggest that Snf1 protein kinase is required for maltose induction in addition to its role in Mig1 repressor inhibition. Loss of Snf1 kinase in a *mig1Δ* background does not enable the doubly deleted strain to grow on maltose in presence of an inducible *MAL*-activator protein. Surprisingly, however, Hu et al (2000) showed that a *snf1Δ mig1Δ* strain carrying a constitutive *MAL*-activator allows constitutive expression of maltase but no maltose transport activity is detected. Furthermore, no significant difference was detected between the levels of maltose permease mRNA in the *SNF1 mig1Δ* [pMAL63/43-c] and *snf1Δ mig1Δ* [pMAL63/43-c] strains. Expression of a *MAL61promoter-LacZ* fusion with the junction site at the 5` AUG of the ORF was unaffected by deletion of *SNF1* in the *mig1*Δ strain suggesting that mRNA stability, nuclear export, and translation initiation are unlikely candidates for the Snf1 kinase function. Hu et al. (2000) concluded that Snf1 kinase is required for maltose permease expression at a post-transcriptional step via regulation of permease synthesis, stability, and/or localization. The results reported in Chapter 1 explore these possibilities.

RESULTS

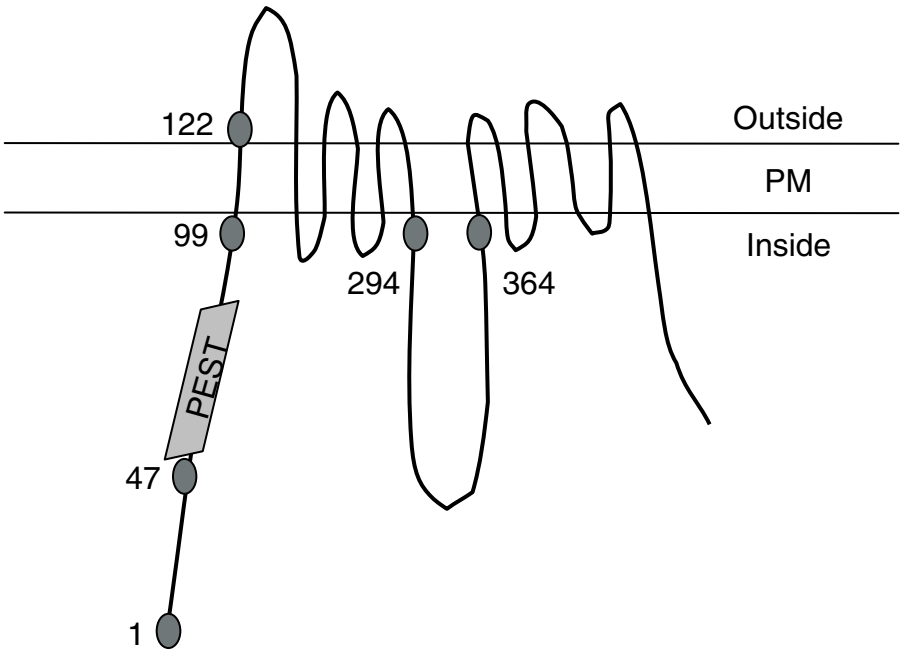
Translation of MAL61 mRNA is not significantly affected in snf1Δ mig1Δ [pMAL63/43-c] strain

The primary effect of glucose repression is at the level of transcription by blocking expression of target genes (reviewed in Carlson, 1999; Johnston, 1999). Reportedly, glucose can affect protein levels by causing degradation of the corresponding mRNA, by increasing the degradation rate of the protein, by inactivating the protein so that it is not functional and, or by causing it to mislocalize (Gancedo, 1998). Control of the mRNA translation rate may not be common in yeast but has been shown in the case of the transcriptional activator Adr1 which is required for expression of *ADH2*, the alcohol dehydrogenase gene. We undertook to distinguish whether *MAL61* mRNA usage was defective in the *snf1Δ mig1Δ* [pMAL63/43-c] strain using *MAL61-LacZ* reporter constructs.

The following series of fusions to *LacZ* were constructed. All constructs included the complete 872 base pair *MAL61-MAL62* bidirectional promoter sequence and the 5` un-translated region (UTR) to the 5` AUG (translation initiation codon) of the *MAL61* open reading frame. The *MAL61* junction sites are as follows: at codon 1, at codon 48 before the start of the N-terminal trafficking signals and the PEST region, at codon 99 just before the first transmembrane domain, at codon 122 just after the first transmembrane domain, at codon 294 just after the sixth transmembrane domain, and at codon 364 just before transmembrane domain seven (Figure 5). Despite repeated efforts, we were unable to construct reporter fusions to codon 548 just after TMD 12 or to the full

Figure5. The structure of *MAL61promoter-LacZ* reporter constructs

Mal61 maltose permease is a member of 12 transmembrane domain family of sugar transporters with the proposed topology as shown (Cheng and Michels, 1989). The position of the *MAL61-LacZ* junction sites in the *MAL61* ORF are indicated by filled ovals (●) and the codon number. The position of the putative N-terminal PEST sequence is also shown.



length *MAL61* ORF. All of the constructs were introduced into *snf1Δ mig1Δ* strain along with a plasmid carrying a constitutive *MAL*-activator gene, *MAL63/43-c*. The transformants were grown in selective medium containing 0.1% glucose and β-galactosidase expression was assayed.

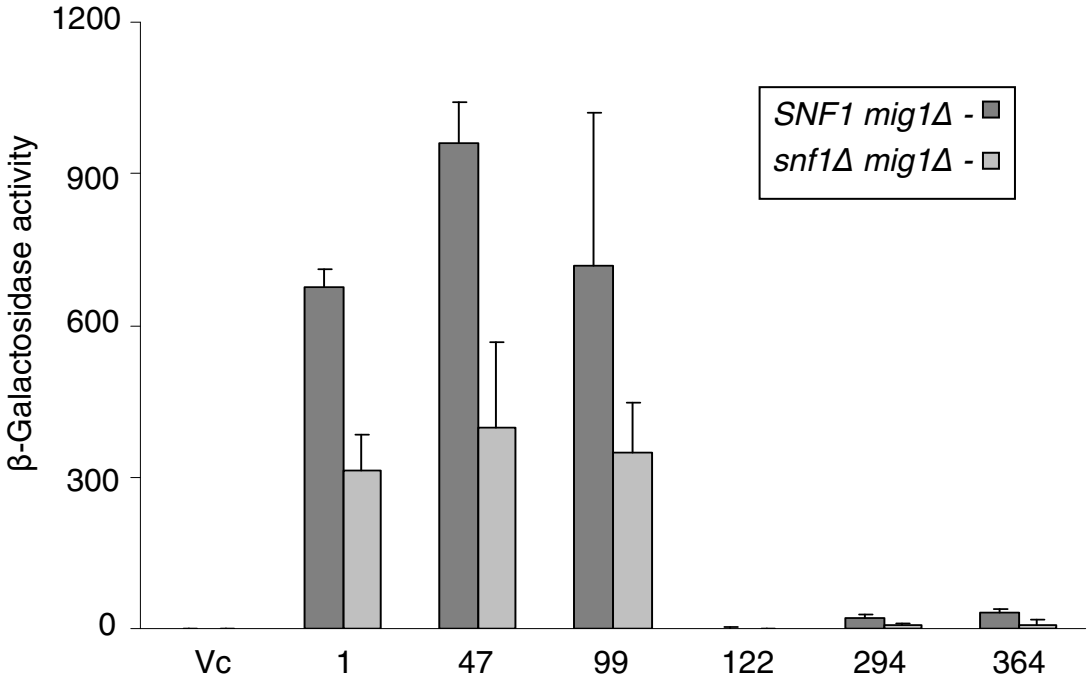
MAL61 mRNA levels were reported to be similar in the *SNF1 mig1Δ* and *snf1Δ mig1Δ* strains (Hu et al., 2000). However, the β-galactosidase activity of the reporter at codon 1 and driven from the *MAL61* promoter suggests that *MAL61* mRNA usage may be slightly defective in the *snf1Δ mig1Δ* strain (Figure 6). Under identical growth conditions, *LacZ* expression is approximately 2-fold lower in the doubly deleted strain compared to the *SNF1 mig1*Δ control. For all of the *MAL61-LacZ* constructs, loss of Snf1 kinase results in *LacZ* expression that is at least 2-3 fold lower compared to the strain carrying only *mig1Δ*.

A sudden loss of β-galactosidase expression is observed once the Mal61p junction site is at residue 122, just after the first transmembrane domain (TMD). Constructs in which the junction site is in a more downstream position in Mal61p do not restore *LacZ* expression to levels comparable to those observed in constructs containing only Mal61p sequences from the N-terminal cytoplasmic domain. This result suggests that the presence of the first transmembrane domain of Mal61 permease is responsible for the sudden loss of *LacZ* expression. It should be noted that the loss of *LacZ* expression occurs both in *SNF1 mig1Δ* and *snf1Δ mig1Δ* strains but is significantly greater in the *snf1Δ mig1Δ* strain.

Taken together, the results reported in Figure 6 suggest that loss of Snf1

Figure 6. Expression of *MAL61-LacZ* reporter constructs in *snf1Δ mig1Δ* versus *SNF1 mig1Δ* strains

Strains CMY101 (*snf1Δ mig1Δ*) and CMY102 (*SNF1 mig1Δ*) were transformed with pUN30/MAL63/43-c, carrying the constitutive *MAL*-activator gene, and YCp355 vector (Hu et al., 1995), or one of *MAL61promoter-LacZ* reporter constructs (YCp355/MAL61/HA-LacZ-1, -47, -99, -122, -294 and -364) described in Figure 1. The numbers indicate the number of the last *MAL61* codon at the junction site to *LacZ*. Transformants were grown to mid-log phase on YNB selective medium lacking uracil and tryptophan and containing 0.1% glucose as carbon source. Vc indicates vector control. β-Galactosidase activity was assayed with whole cell lysates as described in Materials and Methods. The results presented are an average of four individual transformants.


1200
900
600
300
0
β-Galactosidase activity
SNF1 mig1Δ -
snf1Δ mig1Δ -
Vc
1
47
99
122
294
364

kinase has a modest, possible 2-fold, impact on translation initiation of *MAL61* mRNA. More importantly, they reveal that the presence of even a single TMD dramatically decreases reporter gene expression independent of Snf1 kinase. These findings suggest that translation of *MAL61* mRNA may be coupled to the membrane insertion of the nascent permease and which may lead to inefficient translation, abortive translation or destabilization of the protein product. These possibilities are explored below.

snf1Δ mig1Δ [pMAL63/43-c] strain expresses low levels of Mal61 maltose permease protein

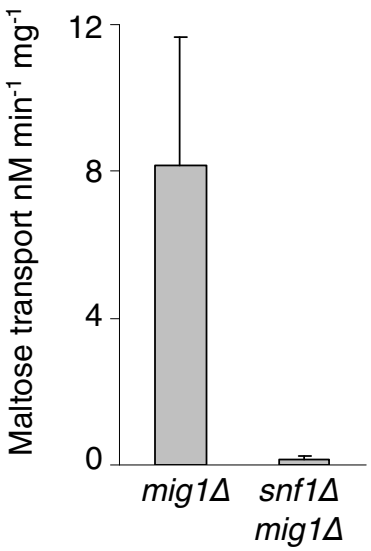
Hu et al. (2000) reported a total loss of maltose transport activity in a *snf1*Δ *mig1*Δ strain carrying a constitutive *MAL*-activator. I utilized an HA-epitope tagged allele of *MAL61* to determine the basis of this lack of transport activity. The *MAL61*/*HA* allele was constructed by (Medintz et al., 1996) and places the HA-epitope at the N-terminus of Mal61p.

SNF1 mig1Δ [pMAL63/43-c] and *snf1Δ mig1Δ* [pMAL63/43-c] strains were transformed with a plasmid carrying *MAL61*/HA. Transformants were grown in selective medium containing 0.1% glucose as the sole carbon source and Mal61/HA protein levels determined by Western blot analysis. 0.1% glucose was shown to be sufficient to allow growth of the *snf1Δ* strain but not to cause glucose repression or inactivation in the *SNF1 mig1Δ* strain (data not shown). Figure 7 shows the results of three independent transformants of both *snf1Δ mig1Δ* and *SNF1 mig1Δ* strains. Mal61p/HA expression in a *snf1Δ mig1Δ*

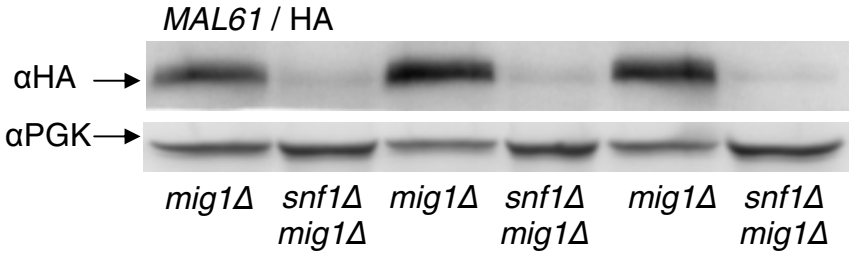
Figure 7. *snf1Δ mig1Δ* [pMAL63/43-c] expresses reduced levels of Mal61/HA protein resulting in low maltose transport activity

Strains CMY101 (*snf1Δ mig1Δ)* and CMY102 (*SNF1 mig1Δ*) were transformed with p413/GPD-MAL63/43-c and pRS315/MAL61/HA. Three independent transformants of each strain were analyzed. Transformants were grown to mid-log phase in selective synthetic medium lacking histidine and uracil and containing 0.1% glucose. Cells were harvested by filtration and used for determination of maltose transport activity (Panel A) or Western blot analysis (Panel B) as described in Materials and Methods. HA-tagged Mal61 maltose permease was detected using anti-HA antibody (Roche) and PGK was detected with anti-PGK (Molecular probes). PGK is used as a loading control and both antibodies were used to probe the same membrane.

A.


Maltose transport nM min-1 mg-1
12
8
4
0
mig1Δ
snf1Δ mig1Δ

B.


MAL61 / HA
αHA
αPGK
mig1Δ
snf1Δ mig1Δ
mig1Δ
snf1Δ mig1Δ
mig1Δ
snf1Δ mig1Δ

[pMAL63/43-c] strain is reduced about 10-folds compared to the amount of maltose permease protein expressed in a *SNF1 mig1Δ* [pMAL63/43-c] strain.

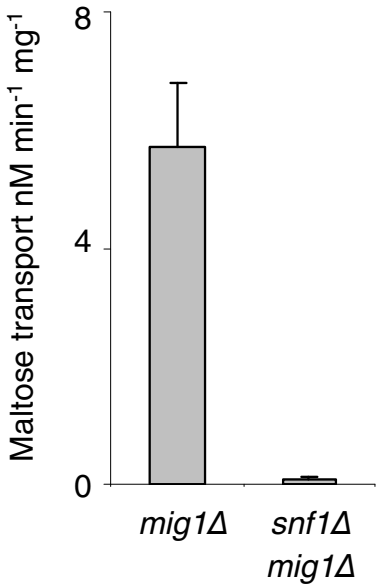
Despite the barely detectable maltose permease protein levels in the doubly deleted strain we attempted to determine its location within the cell. Both *SNF1 mig1Δ* [pMAL63/43-c] and *snf1Δ mig1Δ* [pMAL63/43-c] strains were transformed with a plasmid-borne *MAL61/HA-GFP* fusion gene and the results are shown in Figure 8. Fluorescent signal is observed at the cell surface and in an intracellular compartment, probably the vacuole in the *SNF1 mig1*Δ [pMAL63/43-c] strain. Similar localization patterns were reported by Gadura and Michels (2006), who showed that the vacuolar permease was dependent on endocytosis. In contrast, in the *snf1Δ mig1Δ* [pMAL63/43-c] strain, Mal61/HA-GFP protein does not localize to the plasma membrane. Very low amounts of signal are observed and it was necessary to use increased sensitivity parameters to detect the fluorescence. Additionally, the much reduced amount of permease protein expressed in this strain is observed exclusively in intracellular vesicles or the vacuole. This observation is consistent with the observed poor maltose transport activity (Panel B).

To increase Mal61p/HA expression and to further exclude Snf1 kinase effects on the *MAL61* promoter or *MAL*-activator dependent regulation, we expressed Mal61 permease from a constitutive promoter. *MAL61/HA* was PCR amplified and fused to the high-level GPD promoter (Mumberg et al., 1995), which bypasses the requirement for *MAL*-activator and Snf1 kinase dependent transcription and at the same time increases maltose permease expression

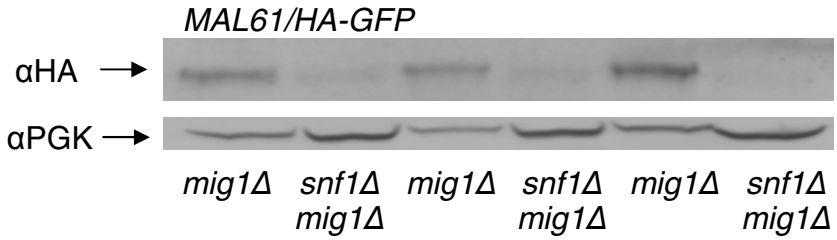
Figure 8. GFP fused maltose permease does not localize to the plasma membrane in *snf1Δ mig1Δ* strain

Strains CMY101 (*snf1Δ mig1Δ*) and CMY102 (*SNF1 mig1*Δ) were transformed with p413/GPD-MAL63/43-c and pUN30/MAL61/HA-GFP, carrying the HA-tagged *MAL61/HA* gene fused to GFP at its C-terminus. Three independent transformants of each type were analyzed. Transformants were grown to mid-log in selective medium lacking histidine and tryptophan and containing 0.1% glucose. Maltose transport activity (Panel A) and Mal61/HA-GFP permease levels (Panel B) determined as described in Figure 7. Mid-log phase cells were used for confocal microscopy and phase microscopy as described in material and methods (Panel C).

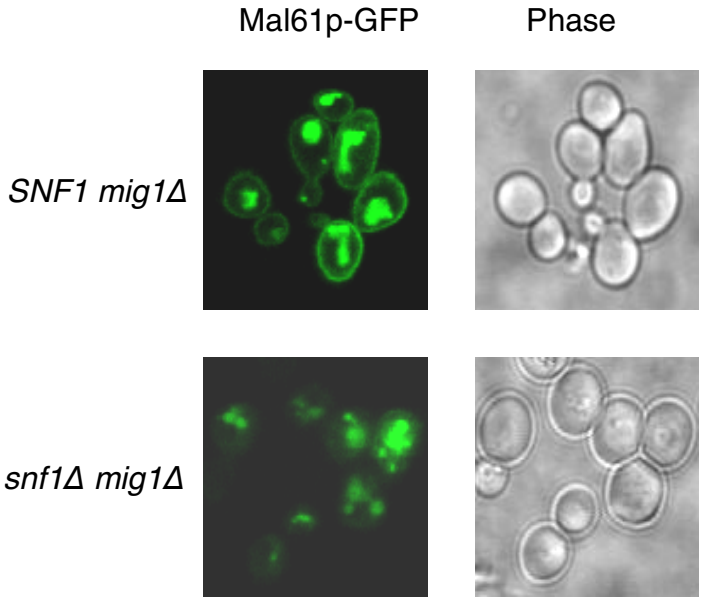
A.


Maltose transport nM min-1 mg-1
8
4
0
mig1Δ
snf1Δ mig1Δ

B.


MAL61/HA-GFP
αHA
αPGK
mig1Δ
snf1Δ mig1Δ
mig1Δ
snf1Δ mig1Δ
mig1Δ
snf1Δ mig1Δ

C.


Mal61p-GFP
Phase
SNF1 mig1Δ
snf1Δ mig1Δ

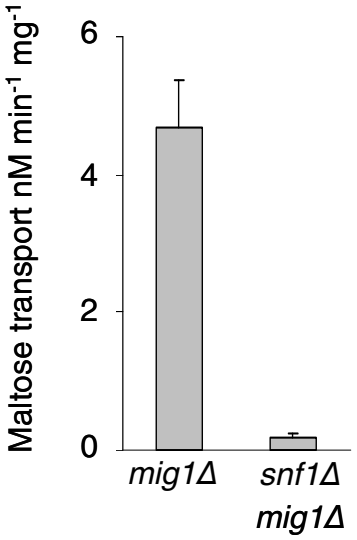
levels. This constitutively expressed *MAL61/HA* was transformed into both *SNF1 mig1Δ* and *snf1Δ mig1Δ* strains and maltose permease protein levels analyzed by Western blot. The results of three independent transformants are shown in Figure 9. While permease expression is higher in both strains, loss of Snf1 kinase still causes dramatically reduced levels of Mal61 permease expression.

These results confirm that maltose permease expression requires Snf1 kinase at post-transcriptional level. In addition, the increased Mal61/HA permease levels allows comparison of Mal61/HA protein stability in the presence and absence of Snf1 kinase. *SNF1 mig1*Δ and *snf1Δ mig1Δ* strains expressing Mal61/HA from the GPD promoter were grown to mid-log phase in selective synthetic medium supplemented with 0.1% glucose. Cyclohexamide (CHX) was added to stop the protein production; cells were collected over the next three hours, total cell extracts prepared, and permease levels determined by Western blot analysis. The results are shown in Figure 10 and are surprisingly similar in the two strains despite the initial difference in protein levels. Both strains show a sharp reduction in protein level during the first hour followed by a slower rate of protein loss over the course of the next two hours. The basis of this bi-phasic degradation is initial vacuolar presence of maltose permease. Nonetheless, the turnover rate of full-length Mal61/HA protein does not appear to be dependent on loss of Snf1 kinase. This result, along with the results reported in Figure 6, suggests the possibility that Snf1 kinase is required during maltose permease translation but after translation initiation.

Figure 9. Constitutive over-expression of *MAL61/HA* increases the protein levels but maltose transport activity remains low

Strains CMY101 (*snf1Δ mig1Δ*) and CMY102 (*SNF1 mig1Δ*) were transformed with p423/GPD-MAL61/HA in which *MAL61/HA* is fused to the constitutive GPD promoter and is carried on a multicopy plasmid. Three independent transformants of each strain were analyzed. Transformants were grown to mid-log phase in selective medium lacking histidine and containing 0.1% glucose. Maltose transport activity (Panel A) and Mal61/HA protein levels (Panel B) were determined as described in Figure 7.

A.


Maltose transport nM min-1 mg-1
6
4
2
0
mig1Δ
snf1Δ mig1Δ

B.

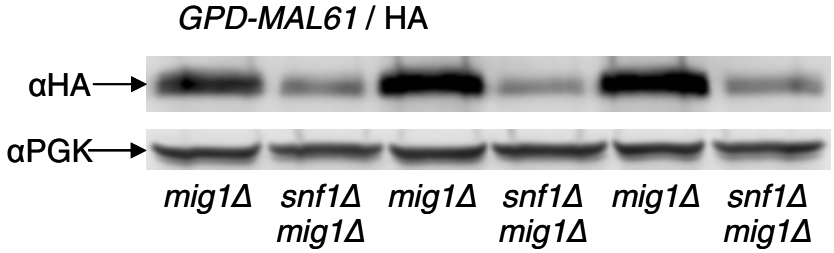
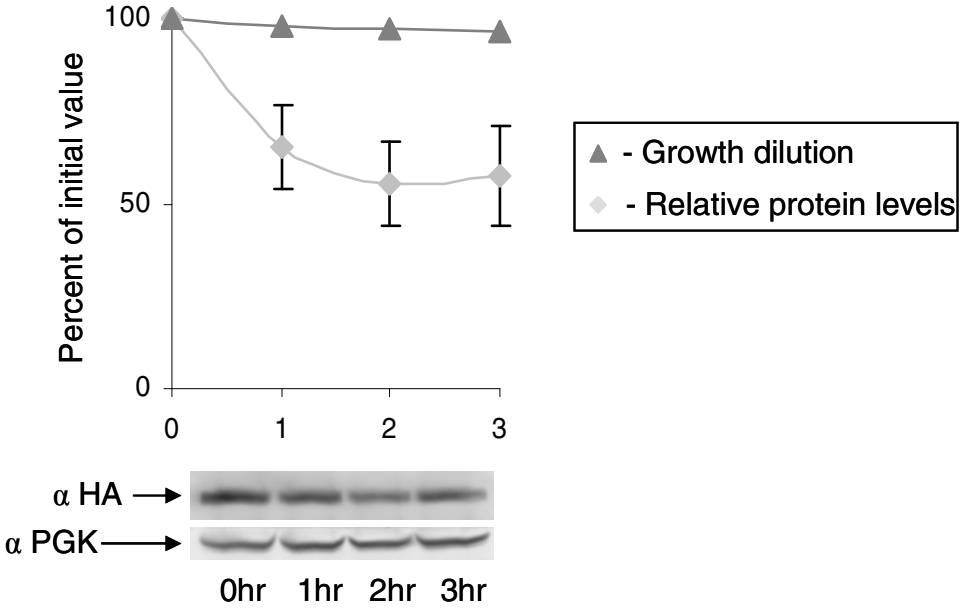

GPD-MAL61 / HA
αHA
αPGK
mig1Δ
snf1Δ mig1Δ
mig1Δ
snf1Δ mig1Δ
mig1Δ
snf1Δ mig1Δ

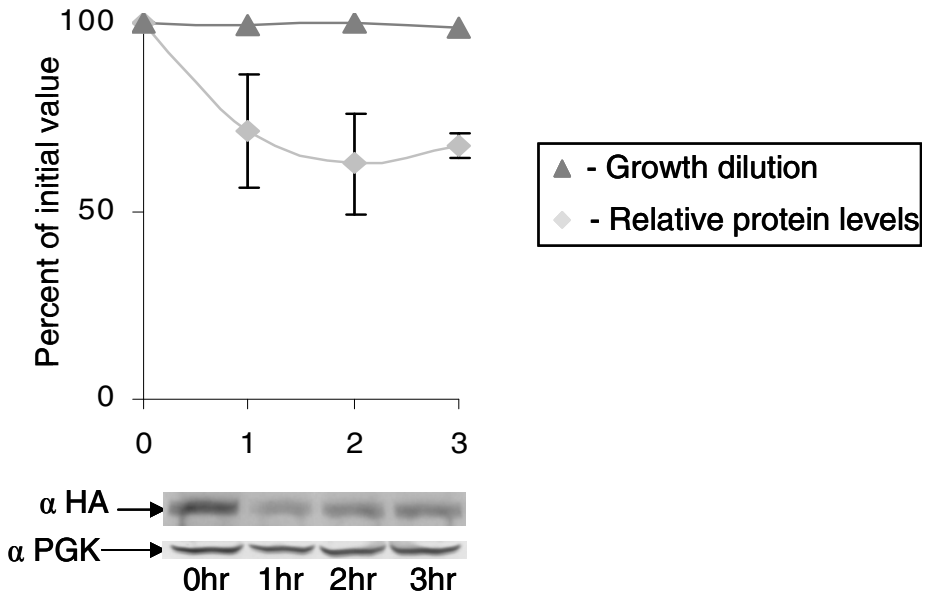
Figure 10. Mal61/HA has a slow turn over rate in the *snf1Δ mig1Δ* strain

Strain CMY101 (*snf1Δ mig1Δ*) and CMY102 (*SNF1 mig1Δ*) were transformed with multicopy plasmid p426/GPD-MAL61/HA. Transformants were grown to mid-log selective medium lacking plus 0.1% glucose. At time 0hr, cyclohexamide was added to a final concentration of 50µg/ml. Samples were removed immediately and hourly for the next three hours. Cells were harvested by filtration, frozen at -80^{o} C, and whole cell extracts prepared for Western blot analysis as described in Figure 7. The results presented are an average of the three independent transformants and a typical blot presented. Growth dilution was determined by dividing the O.D. at time 0 by O.D. at time X. Protein values are represented by diamonds (♦) and triangles (▲) represent the growth dilution.

A. ***SNF1 mig1Δ*** **pGPD- MAL61/HA**



B. ***snf1Δ mig1Δ*** **pGPD-MAL61/HA**



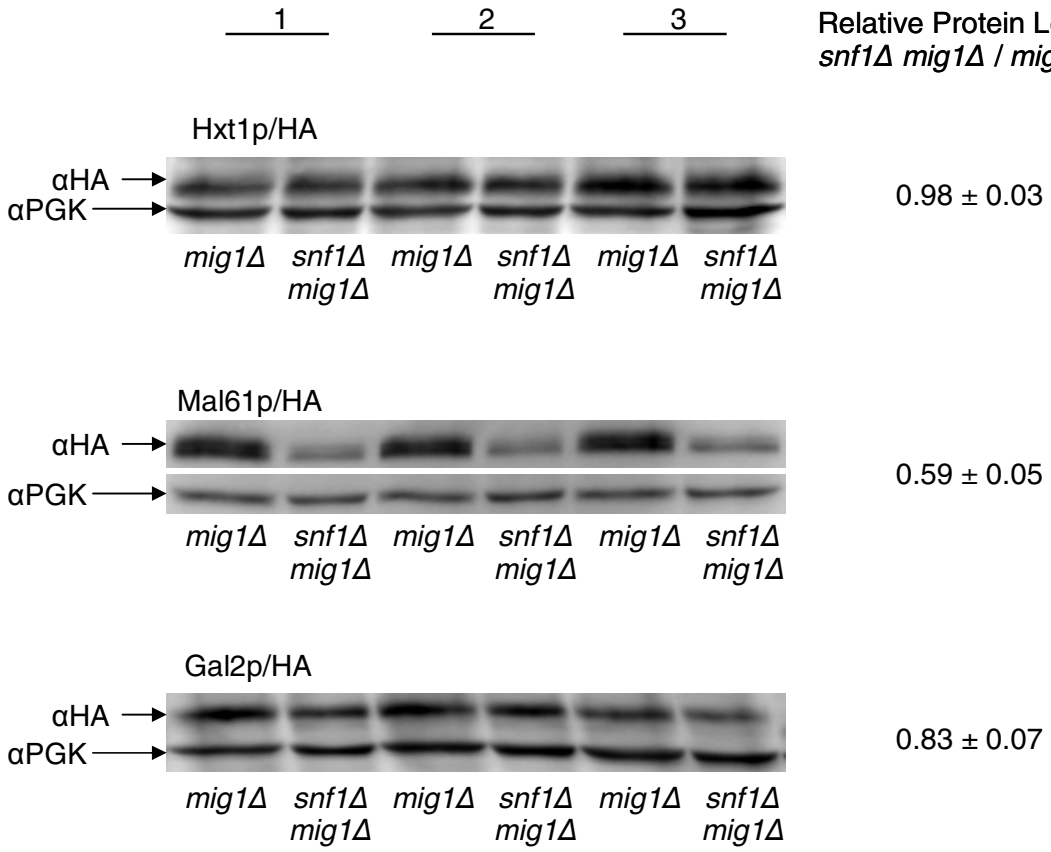
Expression of Hxt1p, the low affinity glucose transporter, and Gal2p, the galactose transporter, are not sensitive to loss of Snf1 kinase

Our results above show reduced levels of Mal61 maltose permease protein expression in the absence of Snf1 kinase. I wanted to determine whether other *Saccharomyces* sugar transporters of the same 12-TMD family as Mal61 permease are also dependent on Snf1 kinase for expression or if the Snf1 kinase dependence is unique to Mal61 permease. *Saccharomyces HXT1* encodes a low-affinity glucose transporter that is expressed by cells grown in high concentrations of glucose, conditions in which Snf1 kinase is inactive (Tomas-Cobos and Sanz, 2002). Therefore, Hxt1p synthesis and expression should not be dependent on Snf1 kinase. *GAL2* encodes the high-affinity galactose transporter (Szkutnicka et al., 1989). Gal2p is 67% identical and 80% similar to Hxt1p and can transport glucose with low affinity. *GAL2* transcription requires *SNF1* (reviewed in (Carlson, 1999; Johnston, 1999). I investigated the affect of loss of Snf1 kinase on Hxt1p and Gal2p expression.

GAL2 and *HXT1* coding regions were amplified by PCR using primers that placed the sequence of the HA-epitope at the N-terminus of the proteins and the tagged gene fused to the constitutive GPD promoter as described in Material and Methods (Mumberg et al., 1995). The plasmid-borne copies of *GPD-MAL61/HA*, *GPD-HXT1/HA*, *GPD-GAL2/HA* were transformed individually into *SNF1 mig1Δ* and *snf1Δ mig1Δ* strains and expression levels of transport proteins monitored by Western blot analysis. The results of three independent transformants of each strain are shown in Figure 11.

Figure 11. Effect of *snf1Δ* on the expression of glucose, maltose and galactose transporter proteins

CMY101 (*snf1Δ mig1Δ*) and CMY102 (*SNF1 mig1Δ*) strains were transformed with one of the following plasmids, p426/GPD-HXT1/HA, p426/GPD-MAL61/HA, or p426/GPD-GAL2/HA. Transformants were grown over night at 30^{o} C in selective medium lacking uracil with 0.1% glucose. Cultures were diluted 2-fold in the morning and allowed to grow for another 3 hours. Cells were harvested by filtration, protein extracts prepared as described in Materials and Methods, and HA expression levels determined by Western blot analysis. Anti-HA antibody was used to detect Hxt1p, Mal61p or the Gal2p. PGK levels were used as loading control. The results shown represent three independent transformants of each strain with each plasmid.


1
2
3
Relative Protein Levels
snf1Δ mig1Δ / mig1Δ
Hxt1p/HA
αHA
αPGK
mig1Δ
snf1Δ mig1Δ
mig1Δ
snf1Δ mig1Δ
mig1Δ
snf1Δ mig1Δ
0.98 ± 0.03
Mal61p/HA
αHA
αPGK
mig1Δ
snf1Δ mig1Δ
mig1Δ
snf1Δ mig1Δ
mig1Δ
snf1Δ mig1Δ
0.59 ± 0.05
Gal2p/HA
αHA
αPGK
mig1Δ
snf1Δ mig1Δ
mig1Δ
snf1Δ mig1Δ
mig1Δ
snf1Δ mig1Δ
0.83 ± 0.07

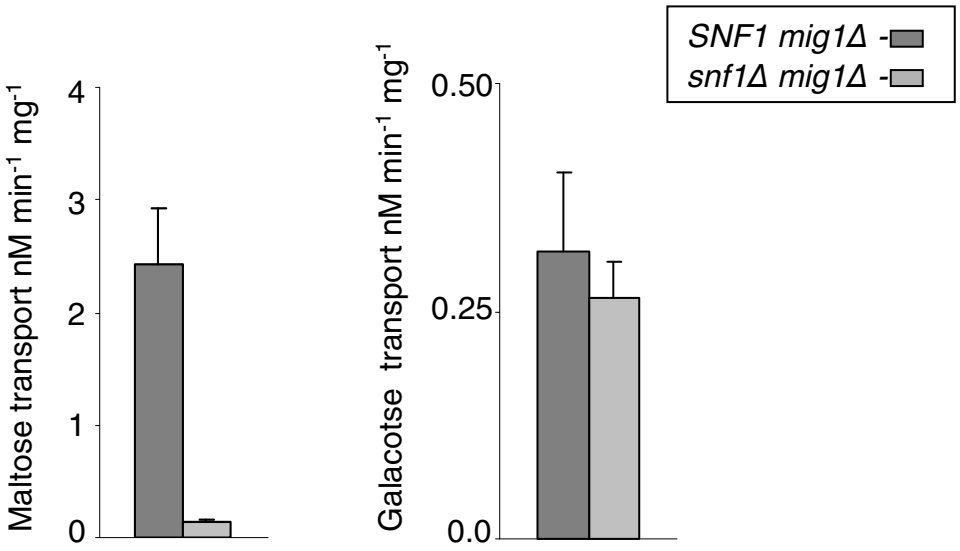
Hxt1 protein levels are similar in both presence and absence of the Snf1 kinase. Thus, as expected, Hxt1p does not require Snf1 kinase activity for its expression and stability. Gal2p expression also is largely unaffected by the loss of Snf1 kinase. This finding is confirmed by measurement of galactose transport in these transformants (Figure 12). These results indicate that the post-transcriptional requirement for Snf1 kinase is specific to maltose permease and is not observed in all 12-TMD sugar transporters including those whose transcription is *SNF1* regulated.

Target of Snf1 requirement is in the N-terminal half of Mal61 permease

Since the galactose transport protein is not sensitive to Snf1p loss, I decided to construct *MAL61-GAL2* and *GAL2-MAL61* hybrids in an effort to localize the region of Mal61p that makes its steady-state level synthesis dependent on Snf1 kinase. Hybrid Mal61 maltose permease and Gal2 galactose transporter proteins were constructed using a PCR based method as described in Materials and Methods. Reciprocal pairs of each hybrid gene were made and, in the figures that follow, the junction site of the hybrid pair is indicated in a cartoon of the topology of these 12-TMD membrane proteins. Expression of all of the hybrid constructs is driven from the constitutive GPD promoter and all are carried on multicopy plasmids. Additionally, the hybrid genes are HA-epitope tagged at the N-terminus to allow detection of the protein by Western blot analysis. Plasmid borne hybrid genes were transformed into *SNF1 mig1Δ* and *snf1Δ mig1Δ* strains and Western blot analysis was carried out to compare hybrid

Figure 12. Comparison of the effect of *snf1Δ* on maltose and galactose transport

Strains CMY101, (*snf1Δ mig1Δ*) and CMY102, (*SNF1 mig1Δ*) were transformed with either p426/GPD-MAL61/HA or p426/GPD-GAL2/HA. Transformants were grown as described in Figure 11 and transport activity determined as described in Materials and Methods. The results shown are an average of three independent transformants of each strain with each plasmid.



protein levels in the two strains.

The first pair of hybrid constructs has the junction sites at the 3` end of transmembrane domain 6 (Figure 13A and 13B). When first half of the Gal2p is fused to the second half of Mal61p, creating a full length 12 TMD hybrid, the resulting protein is expressed at comparable levels both in the *SNF1 mig1*Δ and *snf1Δ mig1*Δ strains (Figure 13B). However, in the reciprocal construct with Mal61p sequence at the N-terminal half, hybrid protein expression is *SNF1*-dependent. Thus, the sequence feature of Mal61p responsible for the low expression levels in the absence of Snf1 kinase lies in the N-terminal half of Mal61p.

In an effort to further localize this sequence, I constructed additional *MAL61-GAL2* fusions with junction sites at various positions in the N-terminal half of the proteins. The second and third pair of constructs were made at the 5` end of the first and third transmembrane domains, as shown in Figures 14A and 15A respectively. The results are not as straight forward as those reported in Figure 13 and, in lieu of the results in Figure 6, are somewhat surprising. Mal61p and Gal2p hybrids that include at least the Mal61p N-terminal domain are very poorly expressed in the *snf1Δ* mutant. The reciprocal hybrid proteins are also sensitive to loss of Snf1 kinase but to a lesser degree. I can not explain the mobility shift observed only in the *snf1Δ mig1Δ* strain expressing the hybrid containing the Gal2p N-terminal cytoplasmic domain (Figure 14B). The mobility is comparable to the mobility of the full-length Gal2p, which runs significantly faster than Mal61p

Figure 13. The target of Snf1 kinase-dependence of Mal61 maltose permease is in the N-terminal half of the protein

Plasmids p426/GPD-M1-293/G264-574/HA and p426/GPD-G1-263/M294-614/HA, carrying reciprocal *MAL61-GAL2* hybrid genes, were used to transform strains CMY101 (*snf1Δ mig1Δ*) and CMY102 (*SNF1 mig1Δ*). The arrow pointing to the filled circle (●) represents the point of sequence swap. The approximate portions of the hybrid belonging to each protein are shown in Panel B. Three individual transformants of each were grown to mid-log phase in selective medium lacking uracil with 0.1 % glucose medium. Fusion protein levels were determined by Western analysis as described in Figure 7.

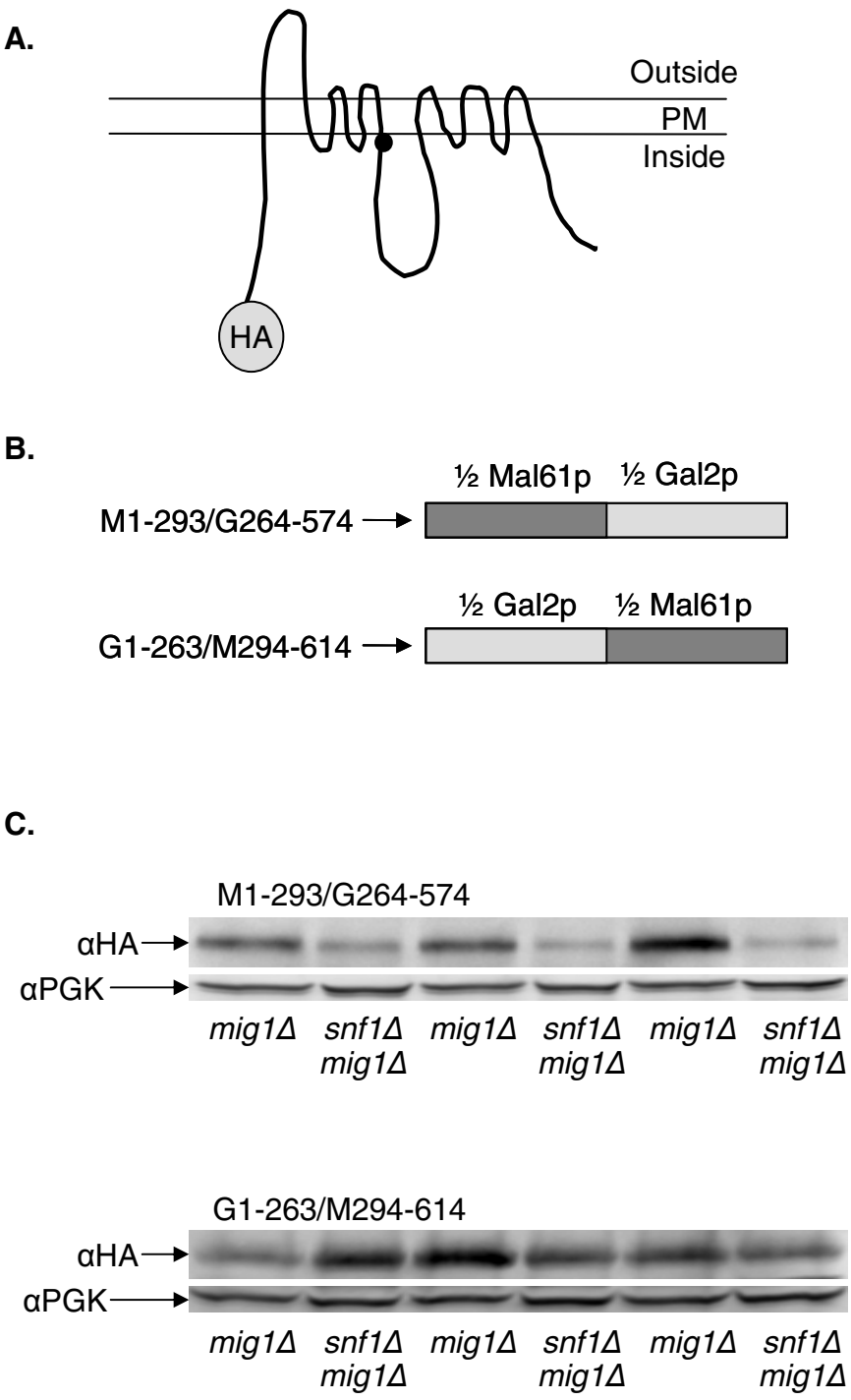

A.
Outside
PM
Inside
HA
B.
½ Mal61p
½ Gal2p
M1-293/G264-574
½ Gal2p
½ Mal61p
G1-263/M294-614
C.
M1-293/G264-574
αHA
αPGK
mig1Δ
snf1Δ mig1Δ
mig1Δ
snf1Δ mig1Δ
mig1Δ
snf1Δ mig1Δ
G1-263/M294-614
αHA
αPGK
mig1Δ
snf1Δ mig1Δ
mig1Δ
snf1Δ mig1Δ
mig1Δ
snf1Δ mig1Δ

Figure 14. Snf1 kinase-dependence of Mal61-Gal2 hybrid proteins with a junction site at the end of the N-terminal cytoplasmic domain

Plasmids p426/GPD-M1-99/G67-574/HA and p426/GPD-G1-66/M100-614/HA carrying *MAL61-GAL2* hybrid genes, were used to transform strains CMY101 (*snf1Δ mig1Δ*) and CMY102 (*SNF1 mig1Δ*). The arrow pointing to the filled cirlcle (●) represents the point of sequence exchange. Panel B shows the final sequence attribution of each of the genes to the hybrid protein (not drawn to scale). Three individual transformants of each were grown to mid-log phase in selective medium lacking uracil with 0.1 % glucose medium. Fusion protein levels were determined by Western analysis as described in Figure 7.

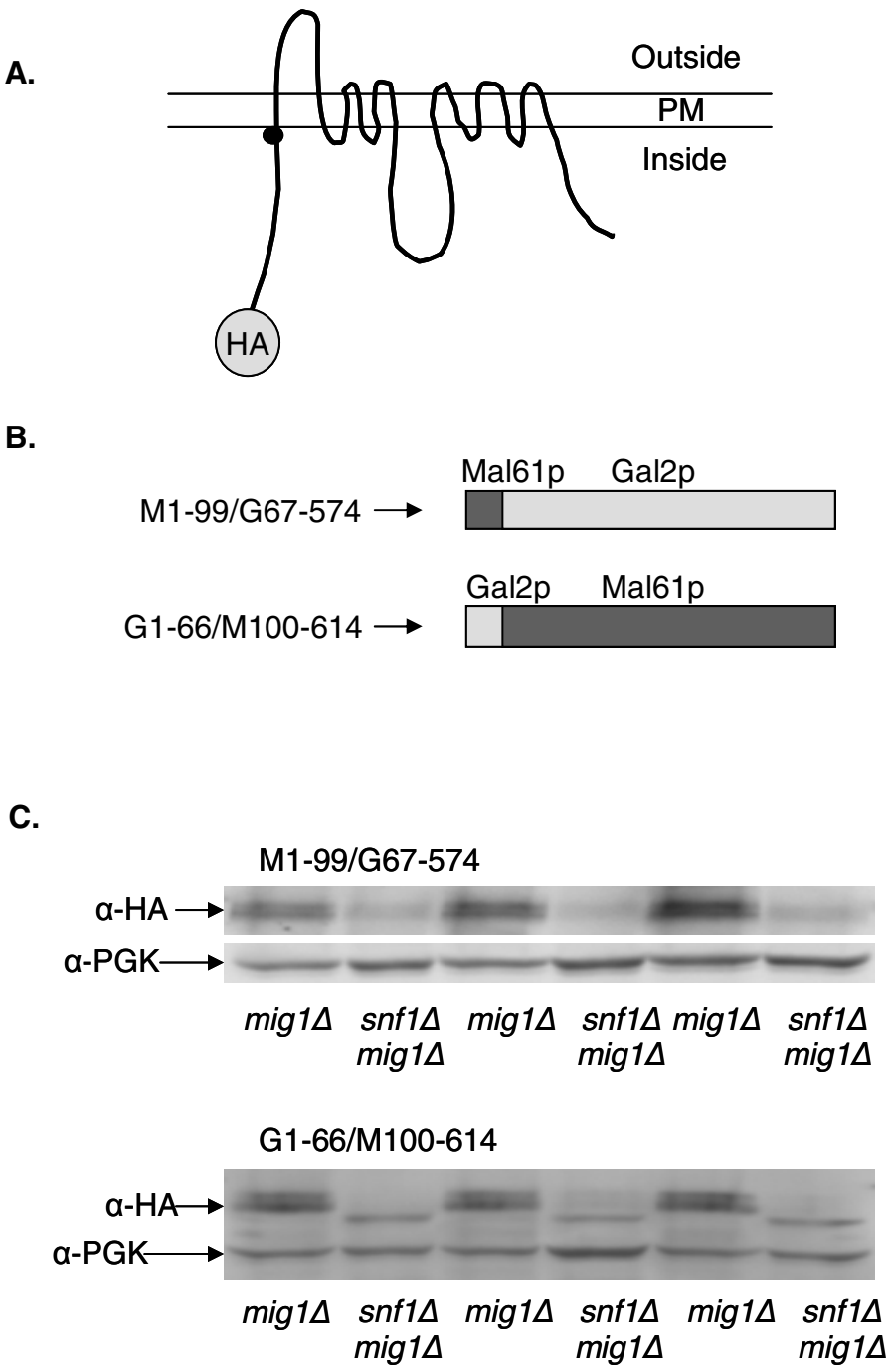
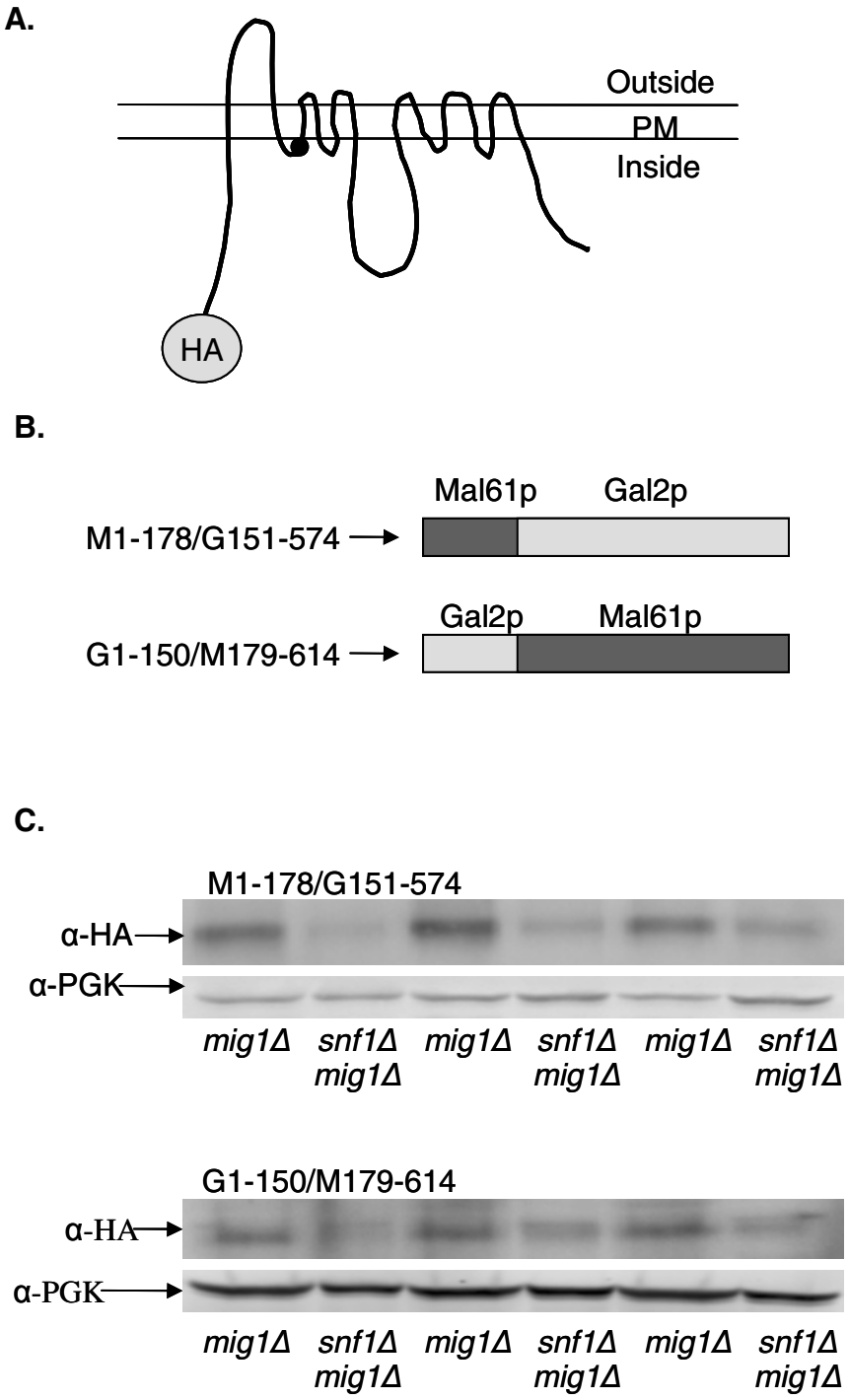

A.
Outside
PM
Inside
HA
B.
Mal61p
Gal2p
M1-99/G67-574
Gal2p
Mal61p
G1-66/M100-614
C.
M1-99/G67-574
α-HA
α-PGK
mig1Δ
snf1Δ mig1Δ
mig1Δ
snf1Δ mig1Δ
mig1Δ
snf1Δ mig1Δ
G1-66/M100-614
α-HA
α-PGK
mig1Δ
snf1Δ mig1Δ
mig1Δ
snf1Δ mig1Δ
mig1Δ
snf1Δ mig1Δ

Figure 15. Snf1p dependence of Mal61-Gal2 hybrid proteins with a junction site after TMD2

Reciprocal hybrid *MAL61-GAL2* constructs with junction site just N-terminal to transmembrane domain 3, were constructed as described in Materials and Methods. The arrow pointing to the filled circle (●) in panel A indicates the position of sequence exchange between the two proteins. Plasmids p426/GPD-M1-178/G151-574/HA and p426/GPD-G1-150/M179-614/HA carrying *MAL61/GAL2* hybrid genes, were used to transform strains CMY101 (*snf1Δ mig1Δ*) and CMY102 (*SNF1 mig1Δ*). Three individual transformants of each were grown to mid-log phase in selective medium lacking uracil with 0.1 % glucose medium. Fusion protein levels were determined by Western analysis as described in Figure 7.


A.
Outside
PM
Inside
HA
B.
Mal61p
Gal2p
M1-178/G151-574
Gal2p
Mal61p
G1-150/M179-614
C.
M1-178/G151-574
α-HA
α-PGK
mig1Δ
snf1Δ mig1Δ
mig1Δ
snf1Δ mig1Δ
mig1Δ
snf1Δ mig1Δ
G1-150/M179-614
α-HA
α-PGK
mig1Δ
snf1Δ mig1Δ
mig1Δ
snf1Δ mig1Δ
mig1Δ
snf1Δ mig1Δ

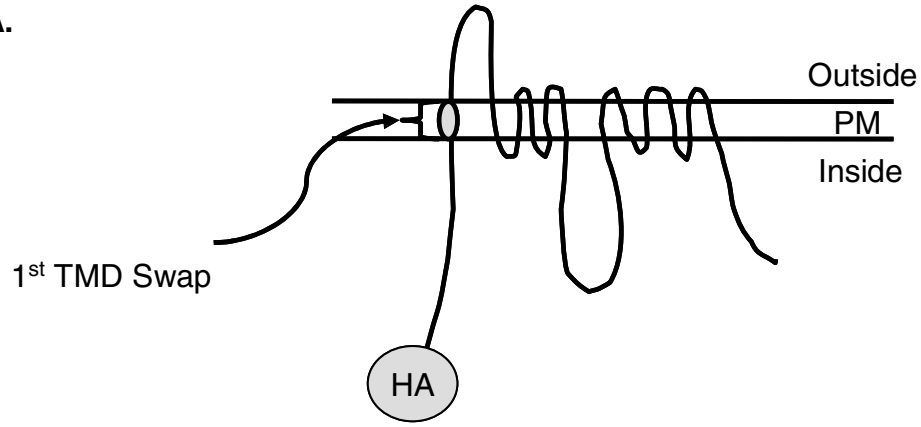
despite the fact that the coding sequences are of similar length, 574 codons for *GAL2* and 614 codons for *MAL61*, but other factors may be in play.

One feature of Mal61p that distinguishes it from Hxt1p and Gal2p is that the first TMD is not especially non-polar. In the Kyte-Dolittle hydropathy analysis reported by (Cheng and Michels, 1989), this TMD barely met the required criteria. Of the 21 residues of the proposed TMD1, seven are polar (including five serines and threonines) and two are charged. These serine/threonine residues may become charged if phosphorylated. In contrast, of the 21 residues of the TMD1 of Gal2p, it contains three polar residues including one each of serine, threonine, and tyrosine and one charged residue (Szkutnicka et al., 1989). We decided to test whether this region alone was responsible for the Snf1p effect. The Mal61p sequence spanning the first transmembrane domain was replaced with that of Gal2p (Figure 12A and 12B). Although the impact of *snf1Δ* is not as severe for the hybrid as for the hybrids in Figures 14 and 15, expression is still significantly lower in *snf1Δ mig1Δ* strain compared to that in the *SNF1 mig1Δ* strain. Taken together these results suggest that residues distributed throughout the N-terminal half of Mal61p that are the target of the Snf1p function but are most especially found in the N-terminal cytoplasmic domain and TMD1.

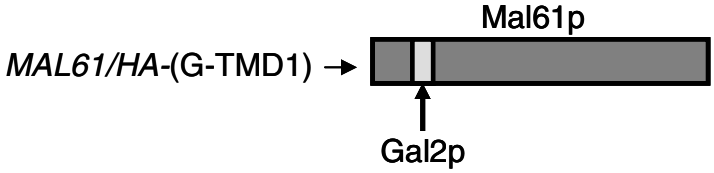
Figure 16. Snf1 kinase-dependence of Mal61p in which TMD1 is replaced with the Gal2p TMD1

Plasmid p426/GPD-MAL61/HA-(G-TMD1) carries the hybrid *MAL61/HA* gene in which the sequence encoding TMD1 has been replaced by the sequence of TMD1 of *GAL2*. The cartoon in Panel A and the diagram in Panel B show the exchanged region. Plasmid p426/GPD-MAL61/HA-(G-TMD1) is transformed into strains CMY101 (*snf1Δ mig1Δ*) and CMY102 (*SNF1 mig1Δ*). Three independent transformants were grown to mid-log phase in selective medium lacking uracil with 0.1 % glucose medium and hybrid protein levels were determined by Western analysis as described in Figure 7. The results are shown in Panel C.

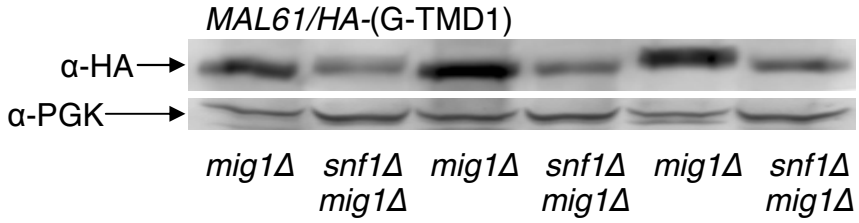
A.


Outside
PM
Inside
1st TMD Swap
HA

B.


Mal61p
MAL61/HA-(G-TMD1)
Gal2p

C.


MAL61/HA-(G-TMD1)
α-HA
α-PGK
mig1Δ
snf1Δ mig1Δ
mig1Δ
snf1Δ mig1Δ
mig1Δ
snf1Δ mig1Δ

DISCUSSION

Snf1 kinase is involved in transcriptional regulation of *MAL* genes by inhibiting Mig1 repressor when cells are cultured in the absence of glucose thereby allowing *MAL* gene induction if inducer (maltose) is present (reviewed in Carlson, 1999). Loss of *SNF1* blocks *MAL* induction because Mig1p can not be inactivated. If this were the only role of Snf1 kinase in *MAL* gene regulation, deletion of both *MIG1* and *SNF1* should restore maltose inducible expression but this is not the case. Hu et al. (2000) discovered that a strain lacking both *MIG1* and *SNF1* is still unable to grow on maltose. They referred to this phenomenon as "glucose inhibition" and showed that it is independent of Mig1 and Mig2 repressors. This result is surprising, because in the case of the *SUC* genes, loss of *MIG1* in a strain lacking *SNF1* is sufficient to restore growth on sucrose (Ronne, 1995). Hu et al. (2000) went on to show that transcription of the *MAL* structural genes encoding maltose permease and maltase was comparable in *SNF1 mig1Δ* and *snf1Δ mig1Δ* strains carrying a constitutive *MAL*-activator (*MAL63/43-c*) gene. Moreover, while maltase expression was unaffected, maltose transport activity was severely reduced in the *snf1Δ mig1Δ* [pMAL63/43-c] strain. Hu et al (2000) concluded that Snf1 kinase was required for a post-transcriptional step in maltose permease expression.

The lack of maltose transport activity results from a dramatic decrease in maltose permease transport protein levels in the strains lacking SNF1

Using an HA-tagged maltose permease protein, I was able to show that

the *snf1Δ mig1Δ* strain carrying a constitutive activator expressed dramatically reduced levels of Mal61p than the isogenic *SNF1 mig1Δ* strain (Figure 7). Overexpression of *MAL61*/*HA* from the constitutive GPD promoter also does not restore maltose transport activity in a *snf1Δ mig1Δ* strain despite increased maltose permease protein levels (Figure 9). Thus, the major reason that *snf1Δ mig1Δ* [pMAL63/43-c] strain does not ferment maltose is that little or no maltose permease protein is detected. But reduced expression is not the sole factor causing the severe defect in maltose transport. Figure 8 shows that the low level of maltose permease expressed in the *snf1Δ mig1Δ* strain does not localize to the plasma membrane but to an intracellular compartment. We are currently investigating whether this intracellular localization originates directly from the Golgi or is by way of the plasma membrane, as is the case for certain *MAL61* mutants with alterations in the N-terminal cytoplasmic domain (Gadura and Michels, 2006). The results will indicate whether Snf1 kinase regulates permease trafficking during synthesis or residency once at the cell surface.

The dramatically reduced Mal61p expression in the *snf1Δ mig1Δ* strain could result from one or more of the following: inefficient mRNA utilization, translation initiation or elongation, and increased rates of permease degradation. I explored these possibilities. Translation initiation control can be exercised at multiple levels including binding of an active ternary complex to the ribosome, association of mRNA with the cap-binding complex, selection of the translational start site, and initiation of polypeptide synthesis (McCarthy, 1998). Glucose depletion is linked to a rapid and reversible inhibition of translation initiation which

does not occur via previously described inhibitory mechanisms such as Gcn2p-dependent phosphorylation or dephosphorylation of eukaryotic initiation factor-2α (eIF-2α) or 4E-BP (an eIF4E-binding protein inhibitor) (reviewed in Sanz, 2003). The mechanism of this inhibition is not understood but is prevented in conditions in which Snf1 kinase is constitutively active, such as *reg1Δ* or *hxk2Δ* mutants strains or galactose growth (Ashe et al., 2000).

I tested the possibility that translation initiation of *MAL61* mRNA is defective in the *snf1Δ* strain. Our *MAL61promoter-LacZ* construct series revealed a mild effect on translation initiation, approximately 2-fold. This result alone can not explain the huge loss in Mal61p expression in the absence of Snf1 kinase. Additionally, based on 5` Rapid Amplification of cDNA Ends (5`-RACE) (data not shown), no significant differences in the 5`-untranslated regions of *MAL61* mRNA from either *SNF1 mig1Δ* or *snf1Δ mig1Δ* strain carrying *MAL63/43*-c were detected. Thus, defects in the initiation of *MAL61* translation do not appear to be a major contributing factor affecting permease synthesis in the absence of Snf1 kinase.

The strains carrying the constitutively expressed maltose permease allowed me to investigate the turn over rate of Mal61p/HA both in presence and absence of Snf1 kinase. The results reported in Figure 10 indicate that the stability of full length maltose permease is unaffected. Thus if degradation contributes to reduced Mal61p expression, it is the nascent Mal61p that must be unstable. Translational control has been demonstrated in the case of Adr1p, the activator required for the expression of *ADH2* gene. (Vallari et al., 1992) showed

that the concentration of Adr1p is 10-fold higher in ethanol grown cells than in glucose grown cells but that there is only a 2-fold difference in the *ADR1* mRNA levels. Since the half life of Adr1p is the same in ethanol or glucose grown cells, the observed difference in protein levels is attributed to reduction in rate of protein synthesis in glucose grown cells. Although the mechanism by which glucose achieves this remains unexplained, this translational control does not depend on the 5` leader sequence, mRNA or protein stability suggesting an affect on elongation (Vallari et al., 1992). More recently Snf1 kinase was shown to physically interact with Adr1p (Young et al., 2002).

Localization studies on GFP-fused maltose permease found that the full-length protein does not localize to plasma membrane and instead is found in intracellular compartments. In *S. cerevisiae*, Snf1 kinase is known to interact with Arf1p and Sec7p (Ho et al., 2002). Arf1p is involved in the regulation of coated vesicle formation and in trafficking within the Golgi while Sec7p is found in the cytoplasm and on Golgi-associated vesicles and is involved in intraGolgi as well as ER-to-Golgi transport (Franzusoff et al., 1991; Stearns et al., 1990).

Taken together, these findings suggest that Snf1 kinase is required for at least two steps in the synthesis of Mal61 maltose permease. First, Snf1 kinase appears to regulate translation elongation of *MAL61* mRNA. This defect could be at the level of ribosomes. Additionally, it could be related to the membrane insertion of the permease, particularly if insertion and translation are coordinated. Second, Snf1 kinase may regulate Mal61 permease trafficking or residency at the plasma membrane.

Snf1 kinase dependency is specific for maltose permease

The results in Figure 11 clearly demonstrate that Mal61 permease, but not Hxt1 glucose transporter or Gal2 galactose transporter, is dependent on Snf1 kinase for efficient expression. Other studies suggest that the lactose transporter of *Kluveromyces lactis* may also be similarly sensitive to loss of Snf1 kinase (Dong and Dickson, 1997; Wiedemuth and Breunig, 2005). Dong and Dickson (1997) reported loss of lactose transport activity in absence of *KlSNF1* under both induced and uninduced conditions. Their findings are comparable to those reported by Hu et al. (2000) for the *MAL* genes. Wiedemuth and Breunig (2005) found that the *K. lactis* lactose transporter encoded by *LAC12* was mislocalized to an intracellular compartment in *snf1Δ* strains. Unfortunately, they did not report lactose permease expression levels in presence or absence of KlSnf1 kinase and so we do not know if protein levels are also reduced. These findings suggest a novel yet well established post-transcriptional function for Snf1 kinase that is preserved in at least two species of yeast.

The Snf1 kinase post-transcriptional dependency for Mal61p expression is located in its N-terminal half

To gain insight into the mechanism of this Snf1 kinase dependency, I tried to localize the region of Mal61p that makes its synthesis dependent on Snf1 kinase. For this I made use of the finding that Gal2p does not exhibit the Snf1p dependency. Maltose permease and galactose permease hybrid proteins were constructed and I demonstrated that the relevant region is in the N-terminal half

of Mal61p. The results in Figure 13 clearly localize the Snf1p-dependent region to the N-terminal half of Mal61p. Our efforts to localize these sequences further were not successful and in fact suggest that the entire N-terminal half of the protein, but particularly the N-terminal cytoplasmic domain and the first TMD, may be responsible. Mal61p N-terminal domain is known to have several regulatory residues required for proper trafficking to and from the plasma membrane. These include the PEST sequence and a dileucine motif (Gadura and Michels, 2006; Gadura et al., 2006; Medintz et al., 2000). Therefore it is not unusual that this region may also harbor residues that are required for Mal61p synthesis and trafficking that are regulated by Snf1 kinase. Additionally, the first TMD of maltose permease is highly unusual in that it has several hydrophilic residues including several serine and threonine residues. It could be this unique feature of its first TMD that sets it apart from Gal2p and Hxt1p.

Integral protein biogenesis begins with the insertion of the protein into the ER membrane. This is known as ER protein translocation and can occur post-translationally, in which protein is completely synthesized in the cytoplasm, or cotranslationally, in which protein is inserted into the ER membrane concomitantly with the peptide elongation (Brodsky, 1998). The Snf1p requirement for Mal61p expression may involve an aspect of translation of this integral membrane protein including the insertion into the ER membrane. This possibility is supported by the dramatic decrease in *MAL61-LacZ* reporter expression when TMD1 is included in the fusion gene (Figure 6). Additional hybrids were constructed exchanging selected regions of the N-terminal halves

of Mal61p and Gal2p. ER associated degradation machinery and/or the unfolded protein response detect any errors during these processes as well as folding. The inaccuracies are either corrected or the protein is sent off for degradation. The corrected version may be sent to the secretory pathway as is shown in the case of Ste6p (Huyer et al., 2004; Loayza et al., 1998). In a large scale identification of integral membrane protein interactions, maltose permease was shown to interact with Cue1p, Gsf2p, and Shr3p (Miller et al., 2005). Cue1p is an ERAD component that recruits Ubc7p to ER where it functions in ERAD (Biederer et al., 1997). Gsf2p is an ER integral membrane protein that promotes synthesis and plasma membrane localization of certain hexose transporters including Gal2p and Hxt1p (Sherwood and Carlson, 1999). Shr3p is an ER packaging chaperone required for incorporation of amino acid permeases into COPII coated vesicles (Gilstring et al., 1999). One or more of these interactions may be important for maltose permease expression and may also require Snf1 kinase. Additionally, Snf1 kinase has been shown to interact with Ssb1p, 2p and Ssa1p, 2p, all members of Hsp70 family (Elbing et al., 2006). Ssb1p,2p are ribosome-associated molecular chaperons involved in folding of newly made polypeptide chains (Pfund et al., 1998). Both Ssa1p and Ssa2p are involved in protein folding and have been implicated in protein translocation and ERAD (Bush and Meyer, 1996). Anyone of these Snf1 kinase functional interactions may be required for efficient translation, translocation, and trafficking of maltose permease. Chapters 2 and 3 of this thesis attempt to determine the mechanism of the Snf1 kinase dependency of Mal61p synthesis.

CHAPTER 2

Involvement of Protein Quality Control Pathways in Mal61 Maltose Permease Expression

INTRODUCTION

The endoplasmic reticulum (ER) processes about a third of all cellular proteins ranging from plasma membrane and secretory proteins to those that are destined to other intracellular organelles such as lysosome and Golgi compartment. ER mediated post-translational modifications vary from folding of the protein to its oligomerization. In addition, the ER is also extremely sensitive to alterations in homeostasis and therefore serves as the major signal transducing organelle in the cell. Upon exposure to different stimuli, ER signals to cytoplasm and nucleus resulting in adjustments for survival. The immediate response is at the level of translation where changes in gene expression ensure long term adaptation (Kaufman, 1999).

Proteins destined for the plasma membrane, such as Mal61p, are translocated into the ER in an unfolded state, and while here they are modified and folded to acquire an active conformation. Quality control systems in the ER ensure the proper folding and assembly of newly synthesized proteins. Accumulation of unfolded proteins results in activation of the Unfolded Protein Response (UPR), which results in increased expression of chaperones to enhance the folding capacity, and Endoplasmic Reticulum Associated Degradation (ERAD) which recognizes and degrades the misfolded proteins (Kaufman, 1999; Kostova and Wolf, 2003). Different but overlapping pathways are used for different classes of proteins. Among the substrates for these pathways can be proteins that are either ER-lumenal or membrane bound and either glycosylated or non-glycosylated (Taxis et al., 2003). Substrate proteins

are recognized, polyubiquitinated, and retrotranslocated into the cytoplasm for degradation via the proteasome. These processes are diagramed in Figure 17 (Ahner and Brodsky, 2004). The retrotranslocon is an aqueous channel consisting of Sec61p, the ubiquitin conjugation enzymes Ubc6p and Ubc7p, and ubiquitin ligase consisting of Hrd3p and Der3p as well as Doa10p. Cdc48p-Ufd1p-Npl4p complex interacts with the polyubuquitinated substrate to pull it through the retrotranslocon and helps in its delivery to the proteasome for degradation (Ahner and Brodsky, 2004; Kaufman, 1999; Kostova and Wolf, 2003; Taxis et al., 2003). Furthermore, additional components, such as Hsp70 chaperones Kar2p and Ssa1p, may be required depending on the substrate. In *Saccharomyces cerevisiae*, mutant forms of Pma1p, Ste6p and CPY* are among the several known ERAD substrates (Loayza et al., 1998; Taxis et al., 2002; Wang and Chang, 2003).

Hu et al., (2000) reported comparable maltose permease mRNA levels in *SNF1 mig1Δ* and *snf1Δ mig1Δ* strains carrying a constitutive activator as well as constitutive expression of maltase. Our recent results reported in Chapter 1 indicate that the low level of maltose transport is due to very low levels of Mal61 maltose permease. We propose that this low Mal61p level results from a combination of the following: a reduced rate of translation initiation, degradation of maltose permease polypeptide during translation elongation, and a block in the trafficking of nascent Mal61p to the plasma membrane. Our studies of *MAL61* and *GAL2* hybrid proteins demonstrate that the *SNF1* requirement for the synthesis/stability of Mal61 maltose permease resides in its N-terminal half

Figure 17. Distinct machinery is required for cytoplasmic and ER lumenal substrates

Lumenal Machinery

Kar2p (Hsp70) – Recognition of ERAD substrate

Ubc1p-Ubc7p-Cue1p – (E2) Ubiquitin conjugation

Der3p-Hrd3 – (E3) Ubiquitin ligase

Sec61p – Retrotranslocon

Cdc48p-Ufd1p-Npl4p complex and Proteasome

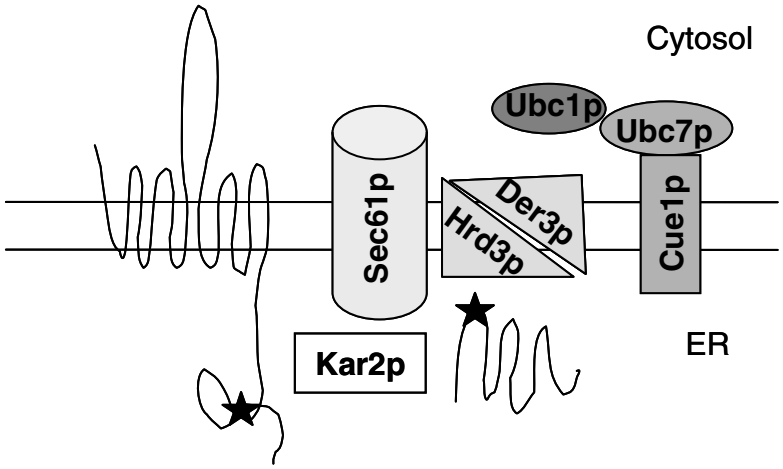
Cytosolic Machinery

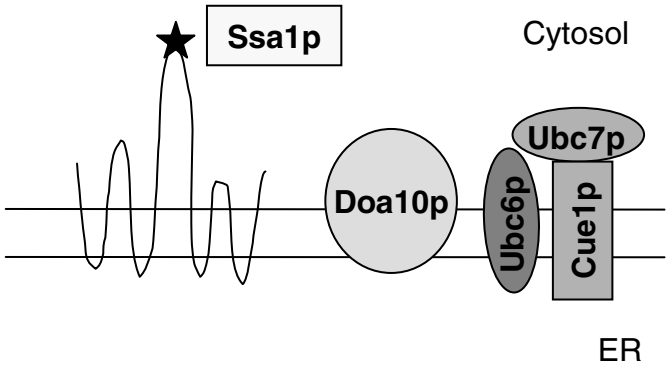
Ssa1p (Hsp70) – Recognition of ERAD substrate

Ubc6p-Ubc7p-Cue1p – (E2) Ubiquitin conjugation

Doa10p – (E3) Ubiquitin ligase

Cdc48p-Ufd1p-Npl4p complex and Proteasome





(Chapter 1, Figure 13). In this chapter, we explore the possibility that in the absence of Snf1 kinase, maltose permease is detained and quickly degraded by protein quality control pathways including UPR, ERAD, and Golgi quality control. To this end, we examined the affect on Mal61p levels in *snf1Δ mig1Δ* strains carrying mutations in ERAD pathway genes or genes encoding components of other protein quality control pathways.

RESULTS

In the absence of Snf1p, maltose permease expression is monitored by the ER quality control pathways

In the previous chapter, I showed that the inability of a *snf1Δ mig1Δ* strain to ferment maltose results from defective expression of Mal61 permease. I demonstrated that this expression defect is post-transcriptional and based on results using *MAL61-LacZ* reporter constructs, is not likely to be due to defects in the translation initiation. Since maltose permease is synthesized on rough ER and ER serves as the initiation point for the secretory pathway, it is likely that ER quality control pathways are involved in controlling synthesis of maltose permease. Thus I decided to investigate the role of ER/Golgi degradation in this Mal61p expression defect.

The accumulation of unfolded proteins in the ER employs two distinct mechanisms. The first is unfolded protein response (UPR) which induces transcription of a large subset of proteins that increase the ER volume or its protein folding capacity. The second is ERAD that promotes the degradation of misfolded proteins (Bukau et al., 2006). In parallel, translation is attenuated to decrease the protein-folding load. This is achieved by phosphorylation of the α-subunit of eukaryotic initiation factor 2 (eIF2α) thereby inhibiting the initiation step in the polypeptide biosynthesis (Ron, 2002). Specific eIF2α kinases have evolved to perform this function, such as Gcn2 kinase that plays this role in the amino-acid starved conditions in yeast.

In yeast, *IRE1* monitors the ER folding capacity and UPR. Ire1p is a

transmembrane kinase that contains a lumenal domain responsible for sensing misfolded forms, a cytosolic kinase, and a ribonuclease domains. In absence of unfolded protein burden, Kar2p, an ER chaperone, binds the lumenal domain of Ire1p thereby limiting its dimerization and activity. Under protein folding stress Kar2p is released which allows Ire1p to dimerize and become active. Once active, Ire1p splices the inactive *HAC1* mRNA thereby allowing its efficient translation. Hac1p enters the nucleus, binds the UPRE, and induces expression of chaperones and UPR and ERAD components (Hampton, 2000).

When soluble lumenal ERAD substrates and some integral membrane substrates fold improperly, it results in their retro-translocation through the Sec61 translocon. However, Sec61p function may be dispensable for other membrane proteins where proteases are suggested to clip the cytoplasmic loops and directly extract the substrate. The delivery of the ERAD substrates to the proteasome is catalyzed by the Cdc48p-Ufd1p-Npl4p complex which binds both the polypeptide backbone and the ubiquitin conjugate: ATP hydrolysis by Cdc48p is required to complete the retro-translocation process (Ahner and Brodsky, 2004). Figure 17 illustrates the distinct degradation machinery for ERAD-lumenal and ERAD-cytosolic substrates.

Although both UPR and ERAD work independently to some extent however, both are strongly interrelated (Hampton, 2000). The UPR pathway induces expression of ubiquitin conjugation enzymes as well as ubiquitin ligases that are involved during ERAD. Since maltose permease synthesis, insertion into membrane, and trafficking begins at the ER, I decided to explore the possibility

that in the absence of *SNF1*, Mal61p becomes a substrate for UPR. I disrupted *IRE1* in the *snf1Δ mig1Δ* strain creating a triply deleted strain and monitored maltose permease protein levels using a construct in which *MAL61/HA* is expressed from the constitutive GPD promoter. Results reported in Figure 18 show significantly increased levels of maltose permease protein in the absence of *IRE1* compared to the *snf1Δ mig1Δ* strain alone. This suggests that the *snf1Δ* mutation leads to maltose permease protein becoming a substrate for the unfolded protein response. Although maltose permease protein levels increase in absence of UPR, maltose transport activity remains low (Figure 18B).

Loss of PEP4 or END3 rescues Mal61p from degradation however, DOA4 does not significantly increase Mal61/HA permease expression in the snf1Δ mig1Δ mutant

Since protein degradation in the yeast cells requires ubiquitination and proteolysis and occurs either via the proteasome or in the vacuole, I wanted to explore the location of Mal61p degradation. Doa4p is ubiquitin hydrolase required for recycling ubiquitin from proteasome-bound ubiquitinated intermediates. It is also involved in general responses to stress, in the degradation of yeast mating factors, in DNA replication, and in the internalization of membrane bound receptors (Wilkinson, 1997). Its absence depletes the levels of free ubiquitin thereby halting further ubiquitination of substrates. Medintz et al. (1998) showed that in a *doa4Δ* strain, glucose-induced proteolysis of Mal61p is severely reduced. Gadura and Michels (2006) show that in a *doa4Δ* strain,

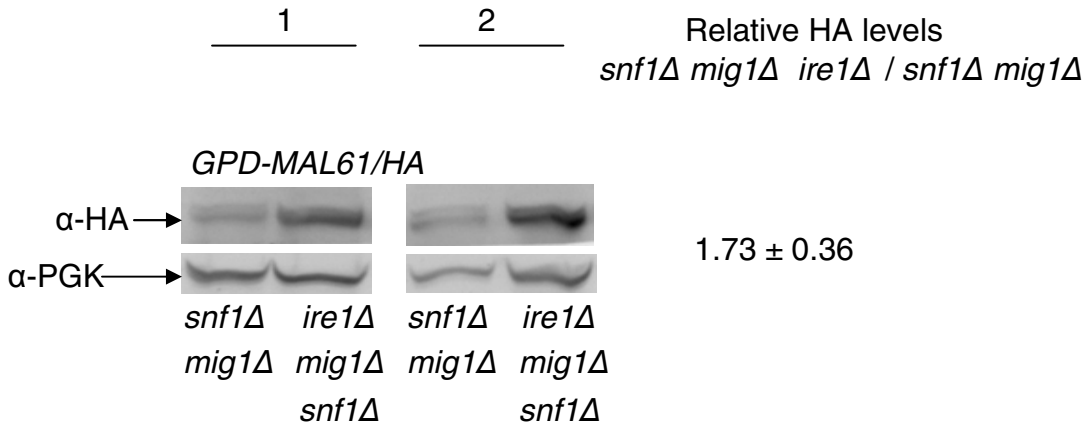
Mal61p localizes to and is slightly enriched at the plasma membrane. Pep4p is a vacuolar aspartyl protease that is required for the posttranslational precursor maturation of vacuolar proteinases (Woolford et al., 1986). In its absence, degradation substrates accumulate in the vacuole. Medintz et al. (1996) showed that glucose-induced proteolysis is dependent on the vacuole and requires *PEP4*. Gadura et al. (2006) found that maltose permease is expressed at normal levels in a *doa4Δ* mutant with no significant increase in maltose transport activity but Mal61p is observed mainly at the cell surface with little seen in the vacuole. Based on these finding, loss of *PEP4* or *DOA4* should lead to increased Mal61/HA permease levels if Snf1 kinase is required to stabilize the permease at a post-translational step, such as during trafficking.

DOA4 and *PEP4* were deleted in the *snf1Δ mig1Δ* strain using the KAN^R and $HYGB^R$ cassettes resulting in triply deleted *snf1Δ mig1Δ doa4Δ* and *snf1Δ mig1Δ pep4Δ* strains (described in Material and Methods). A plasmid-borne copy of *MAL61*/*HA* fused to the constitutive GPD promoter was introduced into each of the strains. Transformants were grown in selective conditions on 0.1% glucose and Mal61/HA permease levels determined by Western blot analysis (Figure 19). The results show that Mal61/HA permease levels are increased in the *snf1Δ mig1Δ pep4Δ* strain. However the *snf1Δ mig1Δ doa4Δ* strain shows only a very minor improvement in the Mal61p/HA levels compared to the *snf1Δ mig1Δ* strain (Figure 19A). Loss of *DOA4* also does not significantly affect maltose transport activity of the *snf1Δ mig1Δ* strain. The *snf1Δ mig1Δ doa4Δ* strain carrying a constitutive *MAL*-activator does not show increased maltose

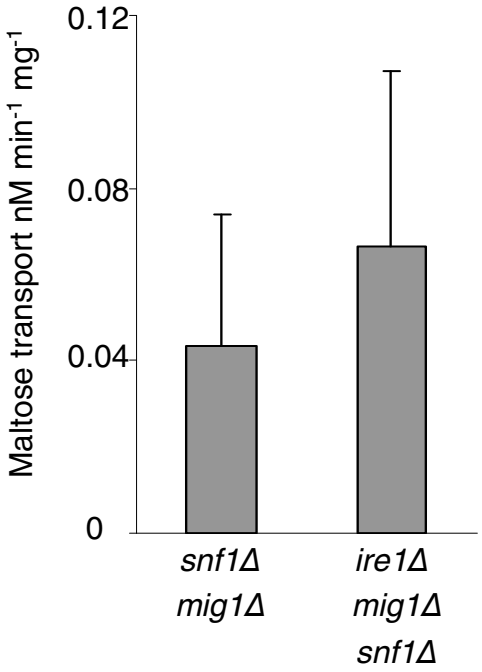
Figure 18. Maltose permease becomes a substrate for UPR in a *snf1Δ* strain

Strains CMY101 (*snf1Δ mig1Δ*) and CMY116 (*snf1Δ mig1Δ ire1Δ*) were transformed with p423/GPD-MAL61/HA. The Western blot results shown are typical for three independent transformants. Cultures were grown to mid-log phase in selective synthetic medium lacking histidine with 0.1% glucose (Panel A). Cells were harvested by filtration, frozen at -80^{o} C, and whole cell extracts prepared for Western blot analysis as described in Materials and Methods. Maltose transport activity was determined for mid-log phase cells as described in Material and Methods (Panel B).

A.


1
2
Relative HA levels
snf1Δ mig1Δ ire1Δ / snf1Δ mig1Δ
GPD-MAL61/HA
α-HA
α-PGK
1.73 ± 0.36
snf1Δ mig1Δ
ire1Δ mig1Δ snf1Δ
snf1Δ mig1Δ
ire1Δ mig1Δ snf1Δ

B.


Maltose transport nM min-1 mg-1
0.12
0.08
0.04
0
snf1Δ mig1Δ
ire1Δ mig1Δ snf1Δ

transport activity (Figure 19B). These results suggest that vacuolar degradation is also a significant factor affecting permease levels.

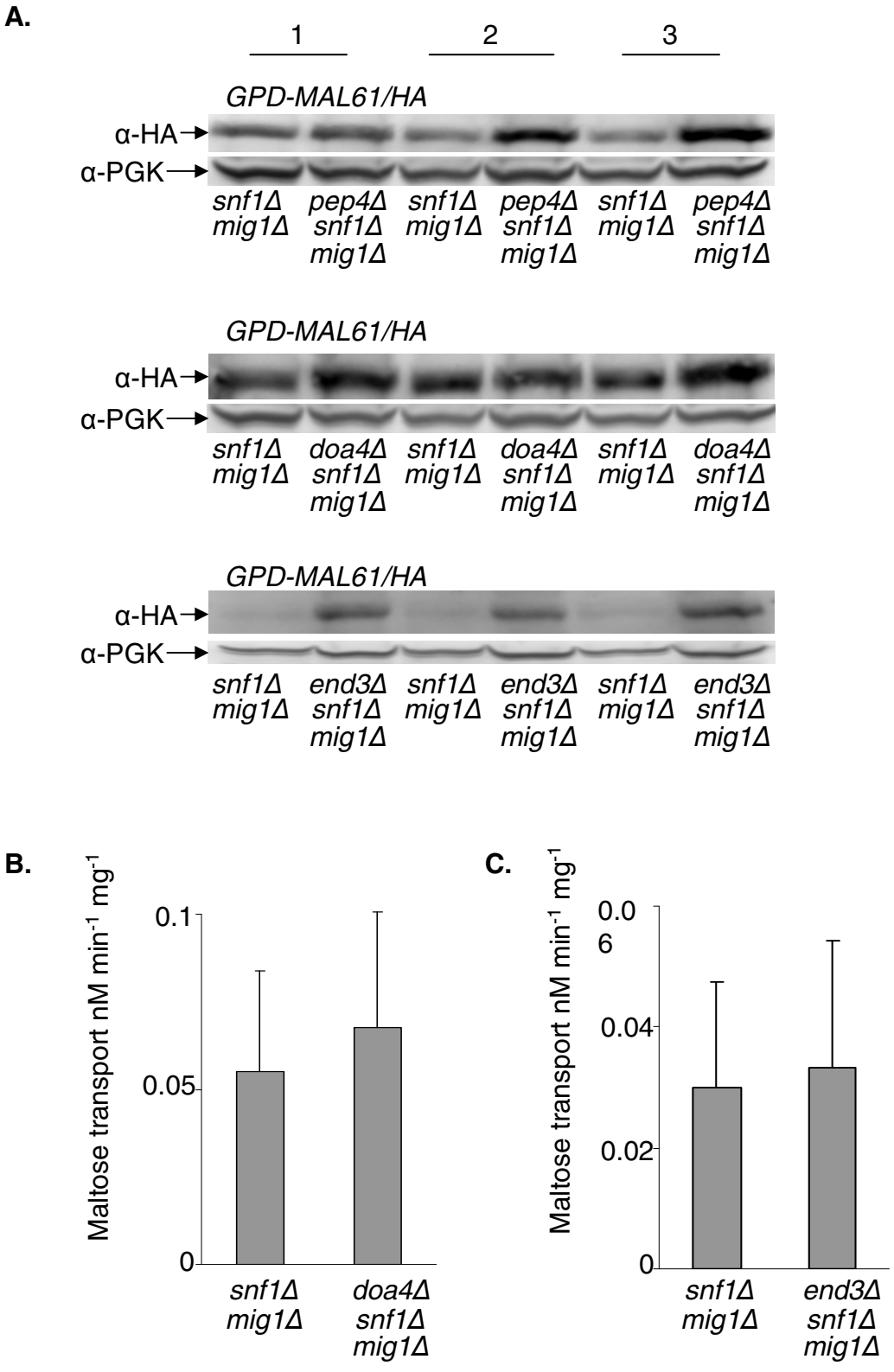
End3p is an EH domain-containing protein involved in endocytosis and actin cytoskeleton organization (Tang et al., 1997). In order to investigate if low levels of Mal61 maltose permease expressed in the *snf1Δ* strains reach plasma membrane or go to the vacuole directly from the ER, I mutated *END3* in the *snf1Δ mig1Δ* strain by one step gene replacement. The triply deleted strain was transformed with *GPD-MAL61/HA* and the resulting transformants were grown in selective conditions in 0.1% glucose. The results reported in Figure 19 show a significant increase in maltose permease protein levels when internalization from the plasma membrane is blocked. These results suggest that in the absence of *SNF1*, Mal61p/HA permease goes to the plasma membrane but is rapidly internalized and degraded in the vacuole. Maltose transport activity however, remains low in the triply deleted strain suggesting that the permease protein is inactive (Figure 19B).

Absence of UBC6 increases Mal61p expression in snf1Δ mig1Δ strain, however, snf1Δ mig1Δ ubc7Δ strain does not show enhanced expression of the permease

Based on the findings described in Figure 18, I decided to investigate the role of ERAD in Mal61/HA peremase synthesis. ERAD substrates include ER lumenal and integral membrane proteins. The particular ERAD machinery used in each of these cases, lumenal versus integral membrane proteins, is unique

Figure 19. Both *pep4Δ* and *end3Δ* reduce Mal61p degradation in the absence of Snf1 kinase whereas *doa4Δ* does not

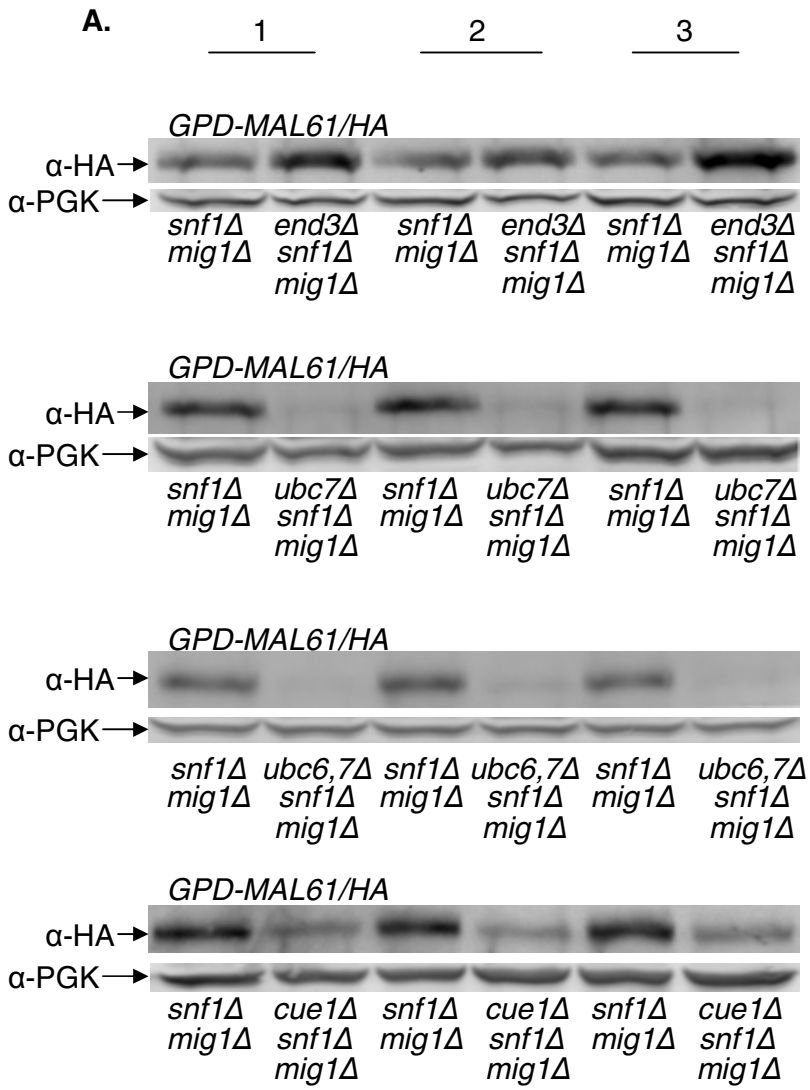
Strains CMY101 (*snf1Δ mig1Δ*), CMY105 (*snf1Δ mig1Δ pep4Δ*) and CMY104 (*snf1Δ mig1Δ doa4Δ*) were transformed with p426/GPD-MAL61/HA. Whereas CMY101 (*snf1Δ mig1Δ*) and CMY (*snf1Δ mig1Δ end3Δ*) were transformed with p423/GPD-MAL61/HA. Three independent transformants of each were analyzed. Cultures were grown to mid-log phase in selective synthetic medium lacking uracil with 0.1% glucose. Western blot analysis was performed as described in Materials and Methods (Panel A). CMY101 and CMY104 were transformed with pUN30/MAL63/43-c. Four independent transformants were analyzed. Mid-log phase cultures were grown in selective medium lacking tryptophan plus 0.1% glucose. Maltose transport activity was determined as described in Materials and Methods.


A.
1
2
3
GPD-MAL61/HA
α-HA
α-PGK
snf1Δ mig1Δ
pep4Δ snf1Δ mig1Δ
snf1Δ mig1Δ
pep4Δ snf1Δ mig1Δ
snf1Δ mig1Δ
pep4Δ snf1Δ mig1Δ
GPD-MAL61/HA
α-HA
α-PGK
snf1Δ mig1Δ
doa4Δ snf1Δ mig1Δ
snf1Δ mig1Δ
doa4Δ snf1Δ mig1Δ
snf1Δ mig1Δ
doa4Δ snf1Δ mig1Δ
GPD-MAL61/HA
α-HA
α-PGK
snf1Δ mig1Δ
end3Δ snf1Δ mig1Δ
snf1Δ mig1Δ
end3Δ snf1Δ mig1Δ
snf1Δ mig1Δ
end3Δ snf1Δ mig1Δ
B.
Maltose transport nM min-1 mg-1
0.1
0.05
0
snf1Δ mig1Δ
doa4Δ snf1Δ mig1Δ
C.
Maltose transport nM min-1 mg-1
0.06
0.04
0.02
0
snf1Δ mig1Δ
end3Δ snf1Δ mig1Δ

and parallel pathways with overlapping components are employed (Taxis et al., 2003). The two pathways share the ubiquitin conjugation enzymes Ubc7p, which is required to recognize and label the misfolded proteins. Ubc6p is employed for cytosolic ERAD substrates and is attached to the cytoplasmic face of the ER via its C-terminus. The Ubc7p is anchored to the same surface via association with Cue1p (Gilon et al., 2000). Cue1p is required to recruit Ubc7p. Loayza et al. (1998) showed that Ste6p-166, a mutant form of Ste6p, is degraded by ERAD machinery. Ste6p-166 is significantly stabilized in a *ubc6, 7Δ* double mutant, which implicates the ubiquitin-proteasome system in the rapid turnover of the mutant protein. In a *doa4Δ* strain, where ubiquitin levels are low and the activity of the proteasome is compromised, the mutant Ste6p is again stabilized. However, Loayza et al. (1998) also showed that Ste6-166 does not enter the secretory pathway even when it is not efficiently degraded. I monitored Mal61/HA permease expression in *snf1Δ mig1Δ* strains mutated individually for *ubc6Δ* and *ubc7Δ* as well as a *ubc6*, *7Δ* double mutant. The Western blot results presented in Figure 20A show that Mal61 maltose permease expression is significantly increased, almost 2-fold in the absence of Ubc6p. Maltose transport activity however remains low, comparable to the *snf1Δ mig1Δ* strain (Figure 20B). Surprisingly, Mal61/HAp levels were reduced even further by the introduction of *ubc7Δ* into *snf1Δ mig1Δ* strain. Moreover, these results indicate that *UBC7* is epistatic (upstream) to *UBC6* because the phenotype of *ubc6*, *7Δ* double mutant and *ubc7Δ* strains are similar. I also monitored maltose permease expression in a *snf1Δ mig1Δ* strain lacking *CUE1*. In a large scale identification

Figure 20. Role of ERAD ubiquitin conjugating enzymes (E2), Ubc6p and Ubc7p, in Mal61 maltose permease expression in absence of Snf1 kinase

Strains CMY101 (*snf1Δ mig1Δ*), CMY106 (*snf1Δ mig1Δ ubc6Δ*), CMY107 (*snf1Δ mig1Δ ubc7Δ*), CMY108 (*snf1Δ mig1Δ ubc6, 7Δ*), and CMY109 (*snf1Δ mig1Δ cue1Δ*) were transformed with p426/GPD-MAL61/HA. Cultures were grown to mid-log phase in selective medium lacking uracil with 0.1% glucose. Three individual transformants of each were analyzed by Western blot analysis as described in Material and Methods (Panel A). Maltose transport activity was determined for CMY101 and CMY106 transformants carrying p426/GPD-MAL61/HA and grown as described above (Panel B).


A.
1
2
3
GPD-MAL61/HA
α-HA
α-PGK
snf1Δ mig1Δ
end3Δ snf1Δ mig1Δ
snf1Δ mig1Δ
end3Δ snf1Δ mig1Δ
snf1Δ mig1Δ
end3Δ snf1Δ mig1Δ
GPD-MAL61/HA
α-HA
α-PGK
snf1Δ mig1Δ
ubc7Δ snf1Δ mig1Δ
snf1Δ mig1Δ
ubc7Δ snf1Δ mig1Δ
snf1Δ mig1Δ
ubc7Δ snf1Δ mig1Δ
GPD-MAL61/HA
α-HA
α-PGK
snf1Δ mig1Δ
ubc6,7Δ snf1Δ mig1Δ
snf1Δ mig1Δ
ubc6,7Δ snf1Δ mig1Δ
snf1Δ mig1Δ
ubc6,7Δ snf1Δ mig1Δ
GPD-MAL61/HA
α-HA
α-PGK
snf1Δ mig1Δ
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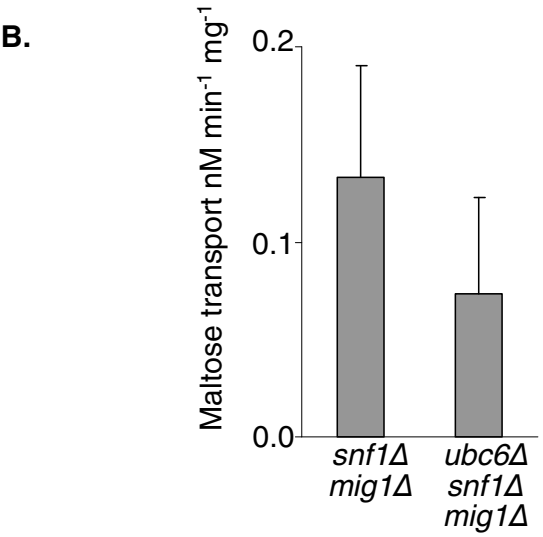
cue1Δ snf1Δ mig1Δ
snf1Δ mig1Δ
cue1Δ snf1Δ mig1Δ


B.
Maltose transport nM min-1 mg-1
0.2
0.1
0.0
snf1Δ mig1Δ
ubc6Δ snf1Δ mig1Δ

of integral membrane protein interactions, maltose permease was shown to interact with Cue1p (Miller et al., 2005). Consistent with the role of Cue1p in Ubc7p recruitment, Mal61/Hap levels are decreased in the triply deleted *snf1Δ mig1Δ cue1Δ* strain (Figure 20A).

An ERAD ubiquitin ligase is involved in maltose permease degradation in the absence of Snf1 kinase

Protein ubiquitination requires the combined action of E2 and E3 enzymes where E2 are ubiquitin conjugating enzymes and E3 ubiquitin ligases. The specific degradation pathway for an ERAD substrate is determined by whether the misfolded lesion is localized to the ER lumen or the cytosol. Consequently, different ERAD machinery components are brought together in various combinations for different substrates. For example, mutant forms of Ste6p* and CPY* employ overlapping but distinct ubiquitination machinery with key differences in their E3 ubiquitin ligase requirement (Huyer et al. 2004). Furthermore, unlike CPY*, the ERAD of Ste6p* does not depend on the proteasome.

Two E3 ubiquitin ligases are known to be involved in the ERAD pathway, Hrd1p/Der3p and Doa10p (Taxis et al., 2003). Der3p acts in a complex with E2 enzymes Ubc7p and Ubc1p and is primarily Ubc6p-independent (Hampton, 2002). Its substrates include CPY*, an ER lumenal protein and Hmg2p, which has ERAD lesion in the lumen of ER. Doa10p, however, functions with both Ubc6p and Ubc7p (Hampton, 2002). Doa10p substrates include mutant forms of

Pma1p and Ste6p both multiple TMD proteins (Loayza et al., 1998; Wang and Chang, 2003). As Ubc6p appears to contribute to the degradation of Mal61/HA in the absence of Snf1 kinase, we hypothesized that Doa10p might be the ubiquitin ligase responsible for maltose permease ERAD. I monitored maltose permease expression in *snf1Δ mig1Δ* strains deleted for either *DOA10* or *DER3*/*HRD1*. Western blot results reported in Figure 21A show a small but significant increase in maltose permease levels in the *doa10Δ* strain. As with the *ubc6Δ*, the *doa10Δ* strain also did not improve the maltose transport activity of the triply *snf1Δ mig1Δ doa10Δ* deleted strain (Figure 21B). The *der3Δ* mutation did not have much impact on maltose permease levels. Huyer et al. (2004) reported that although Doa10p appears to be the major ubiquitn ligase for Ste6p*, Der3p could partially compensate if Doa10p is absent. They concluded that more than one ubiquitin ligase is able to recognize the ERAD substrate, although one is more active than the other. Our results shown in Figure 21 are consistent with this and with the results reported in Figure 20. They suggest that Mal61/HA permease, in the absence of Snf1 kinase, is sensitive to ER-mediated degradation via Ubc6p/Doa10p-mediated ubiquitination. Interestingly, this degradation is enhanced in the absence of ER-localized Ubc7p (Figure 20).

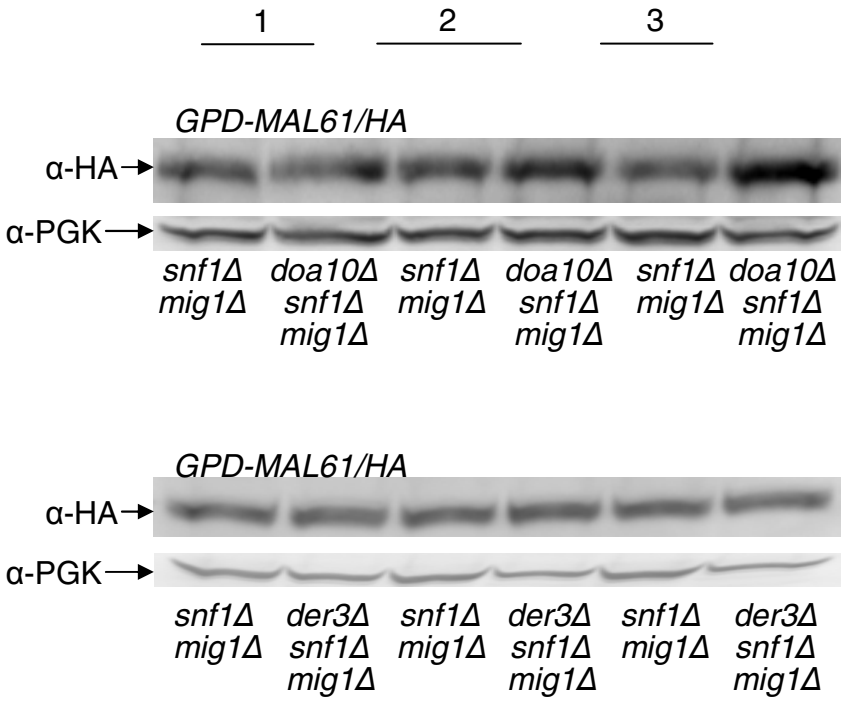
The role of Eps1, Bsd2p, and Pep12p in the maltose permease expression in a strain lacking Snf1p kinase

Taxis et al., (2002) reported that CPY*, a mutant form of carboxy peptidase, is efficiently degraded if it had trafficked between the ER and Golgi

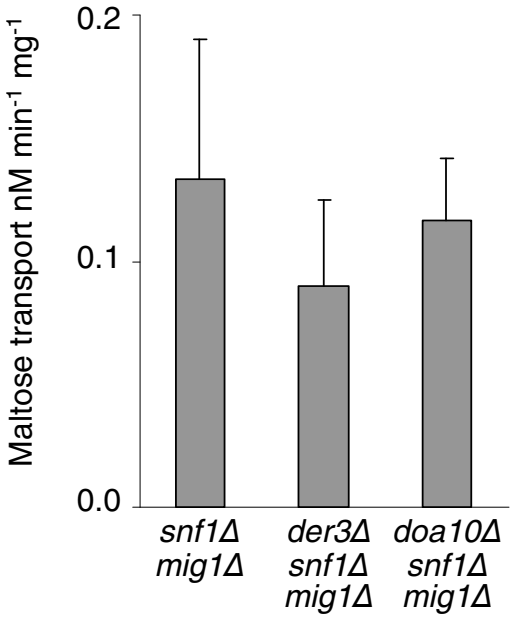
Figure 21. Mal61/HA maltose permease expression in *snf1Δ mig1Δ* strains mutant for the ERAD ubiquitin ligase (E3) genes *DOA10* and *DER3*

Strains CMY101 (*snf1Δ mig1Δ*), CMY114 (*snf1Δ mig1Δ doa10Δ*), and CMY113 (*snf1Δ mig1Δ der3Δ*) were transformed with p426/GPD-MAL61/HA. Three independent transformants of each were analyzed (Panel A). Mid-log cultures grown in selective synthetic medium lacking uracil and 0.1% glucose were used for Western blot analysis (Panel A) and determination of maltose transport activity (Panel B).

A.


1
2
3
GPD-MAL61/HA
α-HA
α-PGK
snf1Δ mig1Δ
doa10Δ snf1Δ mig1Δ
snf1Δ mig1Δ
doa10Δ snf1Δ mig1Δ
snf1Δ mig1Δ
doa10Δ snf1Δ mig1Δ
GPD-MAL61/HA
α-HA
α-PGK
snf1Δ mig1Δ
der3Δ snf1Δ mig1Δ
snf1Δ mig1Δ
der3Δ snf1Δ mig1Δ
snf1Δ mig1Δ
der3Δ snf1Δ mig1Δ

B.


Maltose transport nM min-1 mg-1
0.2
0.1
0.0
snf1Δ mig1Δ
der3Δ snf1Δ mig1Δ
doa10Δ snf1Δ mig1Δ

and was returned to ER upon mutation recognition in Golgi. Wang and Chang (2003) identified Eps1p, a protein related to PDI (protein disulfide isomerase) that is involved in endoplasmic reticulum retention of Pma1-D378N. Pma1p is a plasma membrane H^+-ATPase and in an *eps1Δ* strain, its mutant form escapes ER degradation and moves to the cell surface along with the wild type Pma1p. As established above, Mal61p is also degraded in an ERAD-dependent manner; however, there is a component of maltose permease that escapes degradation. I monitored Mal61/HA expression in a strain lacking *EPS1* gene. In contrast to Pma1p*, Mal61/HA expression was significantly more defective in the *snf1Δ mig1Δ eps1Δ* triply deleted strain compared to the parental strain (Figure 22A).

Hettema et al., (2004) reported a protein quality control mechanism localized in the Golgi. Bsd2p is a three TMD protein that binds to Rsp5p, a ubiquitin ligase, and provides an ubiquitination mechanism for proteins with polar residues within their transmembrane domains. It has been shown to facilitate trafficking of Smf1p and Smf2p metal transporters to the vacuole where they are degraded (Liu et al., 1997). Hettema et al., (2004) suggest a broad role for Bsd2p in quality control of membrane proteins and implicates it in recognition and removal of misfolded membrane proteins. The first TMD of Mal61p is especially polar, as mentioned in Chapter one. In an effort to analyze whether the residual Mal61/HA that escapes ERAD, is captured by the Golgi-quality control in the *snf1Δ mig1Δ* strain, I monitored Mal61p expression in the triply deleted strain. In comparison to the *snf1Δ mig1Δ* strain, maltose permease protein was completely degraded in *bsd2Δ* (Figure 22A).

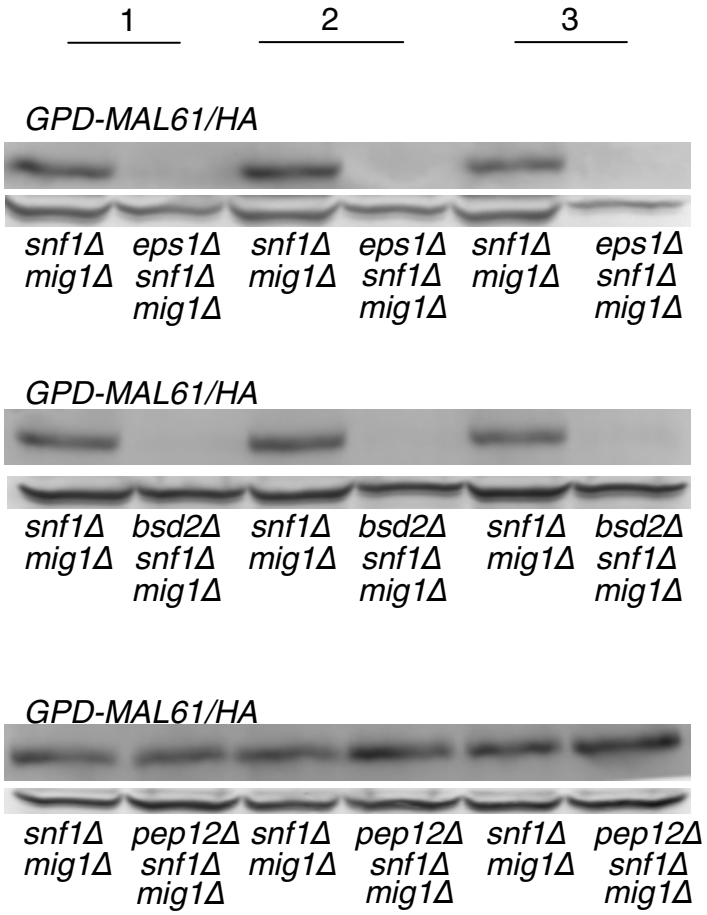
PEP12 encodes the *Saccharomyces* target membrane receptor (t-SNARE) for vesicular intermediates traveling between the Golgi apparatus and the vacuole (Gerrard et al., 2000). In a *snf1Δ mig1Δ* strain lacking *PEP12*, Mal61p/HA expression as well as maltose transport activity are unaffected (Figure 22A, B).

Taken together these results suggest that the ERAD resistant Mal61/HA protein can be degraded by post-ER or Golgi-based quality control pathways. When one quality control pathway is disrupted, others take over to spare the cell from stress.

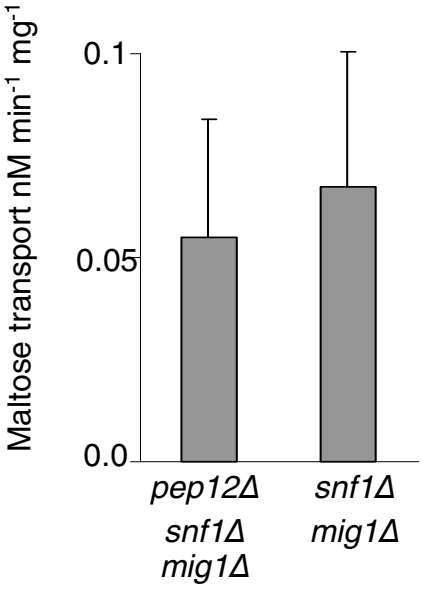
Figure 22. The role of Eps1p, Bsd2p, and Pep12p in maltose permease expression in a *snf1Δ mig1Δ* strain

Strains CMY101 (*snf1Δ mig1Δ*), CMY112 (*snf1Δ mig1Δ eps1Δ*), CMY110 (*snf1Δ mig1Δ bsd2Δ*), and CMY111 (*snf1Δ mig1Δ pep12Δ*) were transformed with p426/GPD-MAL61/HA. Cultures were grown to mid-log phase in selective synthetic medium lacking uracil and 0.1% glucose. Three individual transformants of each were analyzed by Western blot analysis (Panel A) as described in Materials and Methods. Maltose transport activity was determined for CMY101 and CMY111 transformants as described in Material and Methods (Panel B).

A.


1
2
3
GPD-MAL61/HA
snf1Δ mig1Δ
eps1Δ snf1Δ mig1Δ
snf1Δ mig1Δ
eps1Δ snf1Δ mig1Δ
snf1Δ mig1Δ
eps1Δ snf1Δ mig1Δ
GPD-MAL61/HA
snf1Δ mig1Δ
bsd2Δ snf1Δ mig1Δ
snf1Δ mig1Δ
bsd2Δ snf1Δ mig1Δ
snf1Δ mig1Δ
bsd2Δ snf1Δ mig1Δ
GPD-MAL61/HA
snf1Δ mig1Δ
pep12Δ snf1Δ mig1Δ
snf1Δ mig1Δ
pep12Δ snf1Δ mig1Δ
snf1Δ mig1Δ
pep12Δ snf1Δ mig1Δ

B.


Maltose transport nM min-1 mg-1
0.1
0.05
0.0
pep12Δ snf1Δ mig1Δ
snf1Δ mig1Δ

DISCUSSION

Snf1 kinase is required to lift the transcriptional inhibition of *MAL* genes; however, Hu et al. (2000) proposed a post-transcriptional role for Snf1p in maltose utilization. In the previous chapter, I proposed the source of defect in maltose permease expression which results in the inability of *snf1Δ mig1Δ* strain to grow on maltose. Next, I investigate the fate of maltose permease protein in absence of Snf1 kinase. In this chapter, I investigate the possibility that in absence of Snf1 kinase, maltose permease is degraded by protein quality control pathways, particularly UPR and ERAD.

In absence of SNF1, maltose permease becomes a target of protein quality control pathways but increases in maltose permease protein level are not sufficient to restore maltose transport activity

The endoplasmic reticulum tries to provide an optimal environment for processing of secretory and integral membrane proteins but often mistakes arise during this process. The ER employs two distinct mechanisms, UPR and ERAD, for responding to misfolded forms. These two systems are closely linked where UPR induction increases ERAD capacity, loss of ERAD leads to constitutive UPR induction and simultaneous loss of ERAD and UPR may be lethal to the cell (Bukau et al., 2006). The lethality of double mutants means that ERAD is an important alternative to UPR regulation and that the two pathways function independently to some extent: if ERAD were totally dependent on UPR, then loss

of ERAD components in addition to UPR should have no additional phenotypes (Hampton, 2000).

The major regulator of UPR is the transmembrane kinase called Ire1p. I found that deletion of *IRE1* in the *snf1Δ mig1Δ* strain significantly increases Mal61/HA protein levels (Figure 17). This result clearly implicates this quality control pathway as an important factor causing reduced maltose permease protein expression in the absence of Snf1 kinase. This finding suggest that in *snf1Δ* strains the permease is structurally abnormal and is stimulating UPR. The basis of this abnormality remains to be determined. Moreover, the action of Snf1 kinase on maltose permease may be direct or indirect, such as on a maltose permease chaperone. This finding is consistent with the results reported in Chapter 1. Here we found that *MAL61* mRNA translation initiation is largely unaffected by *snf1Δ* (Figure 6) and that the stability of full length Mal61/HA protein in the *snf1Δ mig1Δ* strain is comparable to *SNF1 mig1Δ* strain (Figure 10). Thus, the Snf1 kinase requirement we propose is affecting maltose permease translation elongation, translocation into the ER membrane, or ER/Golgi exit. It should be noted that although Mal61p levels increased in the *snf1Δ mig1Δ ire1Δ* strain, maltose transport activity did not. This suggests yet another level of Mal61p expression that requires Snf1 kinase and implicates trafficking, localization, or functional defects for maltose permease. This suggestion is supported by the *END3* studies described below.

Another component of protein quality control in ER is ERAD. Resulting from the diversity of proteins that fold in ER, ERAD includes different surveillance

mechanisms, each responsible for the degradation of subsets of proteins that share common physical properties. In yeast two parallel but overlapping pathways make up ERAD (Figure 18). The first inspects for proteins that contain misfolded lumenal domains such as CPY*. The second detects misfolded cytosolic domains of transmembrane proteins. Although both of these pathways converge on the ubiquitin proteasome degradation system, they depend on different sets of ER-associated components to detect and deliver misfolded species to the cytosol (Bukau et al., 2006). As mentioned above, the UPR and ERAD are strongly interconnected and induction of UPR employs ERAD to either correctly fold the misfolded protein or degrade it. Since I have already shown that Mal61p becomes stable in the absence of *IRE1*, the next step was to locate the Mal61p misfolded domain (cytosol or lumen of ER) and try to determine which ERAD pathway/machinery is employed.

I mutated various components of ERAD machinery in *snf1Δ mig1Δ* strain and monitored Mal61p levels. Maltose permease levels increased in strains deleted for either *UBC6* or *DOA10* but not *DER3* (Figures 20 and 21). Doa10p and Ubc6p are involved in ubiquitination of proteins that carry cytosolic misfolded domains (Figure 17). A Hsp70 isoform, Ssa1p, is involved in recognition of cytosolic misfolded domains and degradation of integral membrane proteins carrying these defects does not require the retro-translocation machinery (Ahner and Brodsky, 2004). In an affinity capture-mass spectrometric method, Snf1 protein kinase is shown to interact with Ssa1 and Ssa2 heat shock proteins (Elbing et al., 2006). Various studies indicate that although one combination of

ERAD components may be at work, this arrangement does not entirely exclude other ERAD components. My results indicate that in the absence of Snf1 kinase, Mal61p has misfolded cytosolic domain/s that are likely to be recognized by Ssa1p. In turn, the Hsp70 isoform induces UPR and recruits the Doa10p/Ubc6p dependent ERAD (cytosolic) machinery which we show are involved in Mal61/HA degradation in a *snf1Δ* strain. The existence of a second Snf1p-dependent block during trafficking and/or activity is strongly supported by the inability of the above strains to transport maltose (Figures 20B and 21B).

Although Mal61p is ubiquitinated by the Ubc6p and Doa10p complex, absence of *DOA4* which depletes the cell of available ubiquitin does not seem to have a significant affect (Figure19). This is a surprising finding. However, the *snf1Δ mig1Δ* strain lacking *PEP4* showed increased levels of Mal61p suggesting that the full length ERAD-resistant component of Mal61p is degraded in the vacuole. Gadura and Michels (2006) showed constitutive internalization of maltose permease and its localization to the vacuole which is mediated by End3p. In an effort to investigate the route taken by Mal61p to reach the vacuole, I mutated *END3* in a *snf1Δ mig1Δ* strain. The *snf1Δ mig1Δ end3Δ* strain showed enhanced levels of maltose permease protein in comparison to the *snf1Δ mig1Δ* strain (Figure 19). This suggests that some amount of ERAD-resistant Mal61p reaches the plasma membrane and is internalized by endocytosis and degraded in the vacuole in a Pep4p dependent manner. Deletion of *END3* should enrich the level of plasma membrane localized maltose permease (Gadura and Michels, 2006). Since the *snf1Δ mig1Δ end3Δ* strain does not restore maltose transport

activity, I conclude that low levels of plasma membrane localized Mal61p are functionally inactive. Thus, Snf1 kinase appears to be required either directly or indirectly for functional activation of maltose permease.

Hetemma et al. (2004) identified a protein quality control pathway localized to Golgi. Proteins that have polar transmembrane domains are a target for this pathway. Bsd2p is a three-TMD protein that binds the HECT domain ubiquitin ligase Rsp5p and acts as an adapter linking Rsp5p to its substrates. Modification by ubiquitination results in the delivery of substrate from Golgi to the vacuole. This pathway is not only used for down regulation of specific transporters (for example, manganese and iron) but also removal of any abnormal proteins (Hettema et al., 2004). Expression of maltose permease, which has an especially polar TMD1, was even more severely impacted in a strain lacking *BSD2*. This implies that in the absence of Golgi quality control pathway, Mal61p is returned to ER for degradation. In a *snf1Δ mig1Δ* strain lacking *PEP12*, a t-SNARE for vesicular intermediates traveling between Golgi and vacuole, maltose permease expression levels did not change.

Eps1p is an ER resident protein that suppresses the dominant-negative growth phenotype of *pma1-D378N*. Pma1p is plasma membrane H^+- ATPase that has 10 transmembrane domains and is responsible for generating membrane potential. Eps1p is a transmembrane protein known to be involved in disulfide bond formation and retention of ER resident proteins (Wang and Chang, 2003). Its over-expression resulted in ER retention of mutant *pma1-D378N* which was otherwise able to traffic and localize to the membrane. Suspecting that

Eps1p may also play a similar role in Mal61p expression in absence of Snf1 kinase, I monitored maltose permease expression in *snf1Δ mig1Δ eps1Δ* strain. Not only did I get very few *GPD-MAL61/HA* transformants, the maltose permease expression defects were enhanced. The results of the *bsd3Δ* and *eps1Δ* studies are difficult to interpret but suggest that in the absence of these proteins, maltose permease can not escape the ER/Golgi and that this allows it to be degraded more efficiently or inhibit further permease translation.

Taken together these results indicate involvement of various quality control pathways in regulated expression of maltose permease in the absence of Snf1 kinase. Although individual involvement of each of the pathways in Mal61p expression may vary, I propose that in absence of one quality control pathway, others can fully take over. My results indicate that Mal61p requires Snf1 kinase function at a step prior to its exit from ER/Golgi. Another Snf1 kinase function is needed for the rescued Mal61p to transport maltose across the plasma membrane. Therefore, Snf1p is required not only for trafficking but also proper activation of maltose permease.

Enhanced maltose permease degradation in the absence of some protein quality control components suggests translational inhibition

The mammalian cellular response to ER-stress has three main components which include; 1) upregulation of protein processing/degradation capability, 2) repression of protein biosynthesis focused at lowering the load of protein processing, and 3) programmed cell death (Ron, 2002). The decrease in

protein production is accomplished by phosphorylation of eukaryotic translation initiation factor 2 (eIF2α) thereby inhibiting the initiation of polypeptide synthesis (Brostrom and Brostrom, 1998). The mammalian PERK kinase is distantly related to yeast Ire1 kinase. Both have identical mechanisms of activation and PERK is responsible for inhibition of protein synthesis and protein translocation by direct phosphorylation of eIF2α (Ron, 2002).

Two central mediators of stress in yeast are the protein kinases Gcn2p and Ire1p. Yeast eIF2α kinase Gcn2p phosphorylates the alpha-subunit of translation initiation factor eIF2α in response to amino acid starvation. Ire1p is the sensor of UPR. Mammalian kinase PERK has combined the functional properties of yeast Gcn2p and Ire1p (Kaufman, 1999). In yeast, UPR is the first line of defense against ER stress; however translational inhibition has not been shown to be a consequence of UPR.

Several of the ERAD mutants studied here displayed Mal61 permease levels that were significantly lower than those observed in the *snf1Δ mig1Δ* doubly deleted strain. For instance strains lacking *UBC7*, *UBC6* and *UBC7*, *CUE1*, *BSD2*, and *EPS1* all expressed Mal61p at further reduced levels or none at all. These results suggest the possibility that in absence of some ERAD components, there is a feed back to the translational machinery to inhibit protein synthesis/translocation in order to lower the protein processing burden. The Perk-/- mammalian cells are known to lose the ability to control translation in response to ER stress and induction of UPR is delayed significantly (Ron, 2002). In absence of *IRE1*, the *snf1Δ mig1Δ* strain not only expressed Mal61p but the

expression level was comparable to that observed in *SNF1 mig1Δ* strain (Figure 18). Since *IRE1* is the sensor of UPR and inducer for ERAD in yeast, it is possible that some down stream target of Ire1p, perhaps Gcn2 kinase, is responsible for translational inhibition by phosphorylation of translational machinery.

In conclusion, the data so far suggests that Snf1 kinase is required for the successful translation and/or processing of maltose permease in the ER/Golgi system. The results presented in this chapter implicate Snf1 kinase directly or indirectly, in allowing nascent maltose permease to form a native properly folded structure with full functional activity and capable of exiting the ER/Golgi for the plasma membrane. In the absence of Snf1 kinase, most of the nascent maltose permease is degraded by the ER/Golgi quality control systems and what little full-length permease protein escapes from the Golgi is functionally inactive or has a significantly reduced residency at the plasma membrane and is rapidly internalized for vacuolar degradation. This could indicate that Snf1 kinase is needed at several distinct steps in maltose permease synthesis. Alternatively, it is possible that Snf1 kinase is required for a single early but crucial event in permease synthesis that, if defective, stimulates pleiotropic defects in permease synthesis. Chapter 3 explores these two possibilities by attempting to isolate multicopy suppressor of the *snf1Δ* defect.

CHAPTER 3

Multicopy Suppressors of Maltose Non-fermenting Phenotype of *snf1Δ mig1Δ* [pMAL63/43-c]

INTRODUCTION

The results reported in Chapter 1 and 2 show that *Saccharomyces* strains carrying *snf1Δ* expresses only very low levels of Mal61/HA maltose permease protein despite constitutive transcription and stable mRNA levels of *MAL61/HA* (Hu et al., 2000). Moreover, this low level of permease protein is functionally inactive. The effect of *snf1Δ* on permease synthesis is pleiotropic. Mal61/HA permease becomes a target of the ER/Golgi quality control pathways and the protein that escapes degradation by these pathways and reaches the plasma membrane is rapidly internalized and degraded in the vacuole.

The mechanism(s) by which loss of Snf1 kinase causes these defects in permease synthesis is not known. To date, most studies of Snf1 kinase indicate roles in regulating transcription initiation by acting on repressor, activators, and components of the general transcription initiation complexes (Kuchin et al., 2000). In these studies, Snf1 kinase appears to act directly on the regulator. In our study, it is not known whether Snf1 kinase acts directly on maltose permease or on post-transcriptional regulators of permease synthesis. Additionally, it is not clear whether the lack of Snf1 kinase causes a single defect in permease synthesis with pleiotropic effects on stability and transport activity or alternatively whether Snf1 kinase is required at several independent steps in Mal61/HA permease synthesis.

In an effort to answer these questions, I decided to isolate multicopy suppressors of the maltose non-fermenting phenotype of a *snf1Δ mig1Δ* [pMAL63/43-c] strain. Using this method, one might expect to find functionally

related genes whose products are capable of carrying out the same or similar functions as Snf1 kinase. Since there are no known homologues of *SNF1* in *S. cerevisiae*, it is more likely that the isolated suppressors that by-pass the need for Snf1p will be obtained. If Snf1 kinase is required at several independent steps, this approach may not be productive.

RESULTS

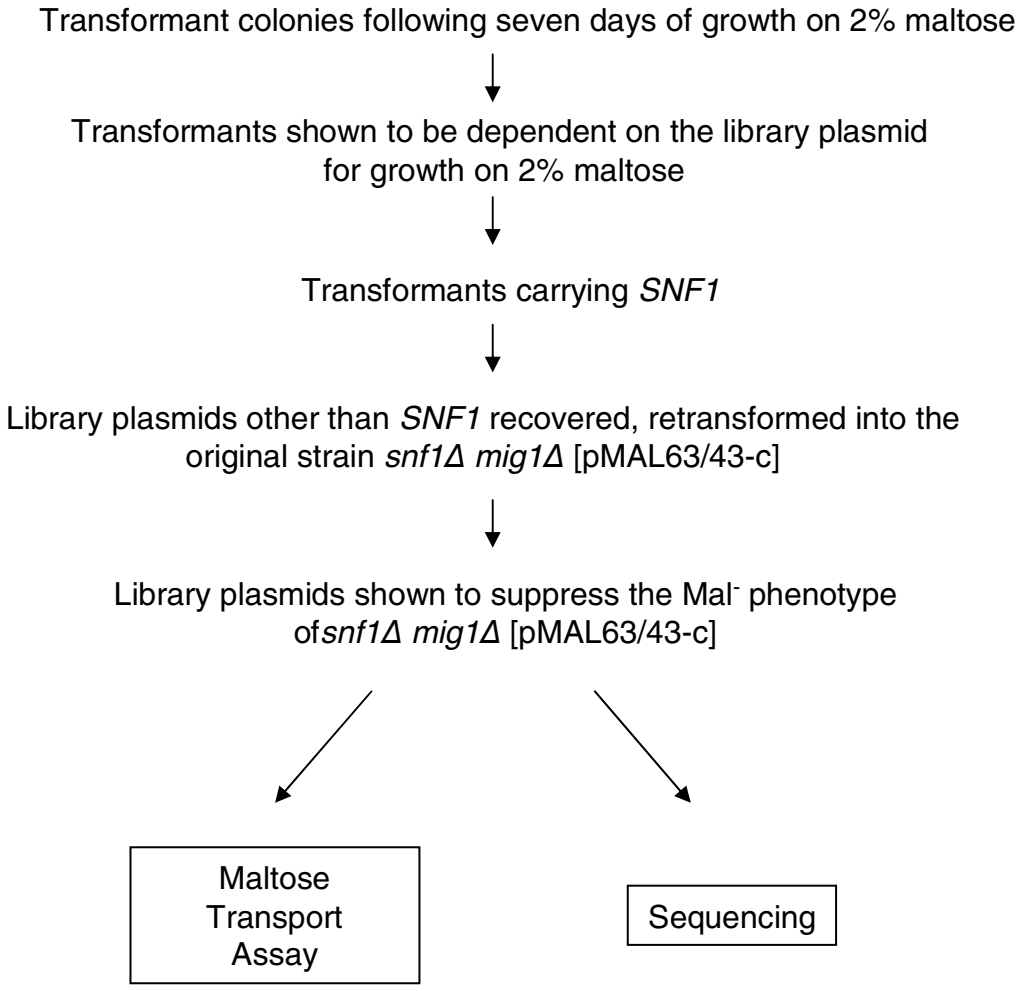
Isolation of multicopy suppressors of snf1Δ mig1Δ [pMAL63/43-c]

The genomic library used to transform *snf1Δ mig1Δ* [pMAL63/43-c] was constructed by Dr. Marian Carlson and is described in Materials and Methods. Random genomic fragments of approximately 10-20Kb were cloned into the 2 micron yeast-*E-coli* shuttle vector YEp24 that carries the *URA3* selection gene. Yep24 is maintained at about 50 copies per cell.

The multicopy YEp24 genomic library was introduced into *snf1Δ mig1Δ* [pMAL63/43-c] strain and plated on selective minimal medium lacking uracil and tryptophan plus 2% maltose. Colonies that formed after seven days incubation at 30^{o} C were repurified on the same medium. The library plasmid was dropped from these transformants by growth in the presence of uracil and the suppressed phenotype retested. The Uracil^{-} transformants that were still Mal^{+} were discarded because the suppression was not dependent on the library plasmid.

The putative suppressor strains that remained were screened by PCR using primers internal to the genomic *snf1Δ*-10 carried by the parental strain and 15 were shown to contain *SNF1*. This suggested that at least fifteen genomes were screened in the search for multicopy suppressors. The library plasmids from the remaining Mal^{+} transformants were recovered in *E. coli,* retransformed into the original *snf1Δ mig1Δ* [pMAL63/43-c] strain, and the Mal phenotype of the transformants determined. Of the plasmids tested, 7 were shown to restore the Mal^{+} phenotype. All of this was done by Ms. Yingzi Yue. Figure 23 outlines the approach taken to isolate the suppressors.

Figure 23. Selection scheme used to isolate multicopy library plasmids that suppressed the Mal$^-$ phenotype of *snf1*Δ *mig1*Δ [pMAL63/43-c]



Six different chromosomal regions were represented among the 7 plasmids as revealed by sequencing of vector/ insert junction sites using primers designed from YEp24 sequence flanking the MCS (done by Ms. Yingzi Yue) (Figure 24). Multicopy suppressor plasmids 8 and 27 contain *MSI1* in common. The *MSI1* gene was previously identified as a multicopy suppressor of sporulation and raffinose utilization defects in *snf1Δ* strains (Hubbard et al., 1992). The Msi1 protein is a subunit of chromatin assembly factor I and negatively regulates the RAS/cAMP pathway via sequestration of Npr1p kinase (Johnston et al., 2001; Ruggieri et al., 1989).

The suppressor plasmids of interest to us were those that restored maltose transport activity. Maltose transport activity was determined for each of the *snf1Δ mig1Δ* [pMAL63/43-c] strains carrying the Multicopy Copy Suppressor (MCS) plasmids, YEp24 vector (negative control), and *SNF1* carried on YEp24 (positive control). Transformants were grown to mid-log phase in selective synthetic medium containing 0.1% glucose and then transferred to the same selective synthetic medium containing 2% maltose for six hours to enhance maltose permease expression. The maltose transport activity of the transformants carrying the multicopy suppressor plasmids is shown in panel A of Figure 25. Only transformants carrying plasmids 25, 31, 54, 56, and 59 exhibited an elevated rate of maltose transport activity and these were selected for further study. MCS8 and MCS27, both of which contain *MSI1*, did not increase maltose transport. These appear to suppress growth by a pathway other than increasing maltose uptake and were not investigated further. The *snf1Δ mig1Δ* [pMAL63/43-

Figure 24. The genomic region contained by the MCS plasmids

The genomic region of the seven library plasmids that allowed *snf1*Δ *mig1*Δ [pMAL63/43-c] to grow on 2% maltose are shown. Sequencing of vector/insert junction sites using primers designed from YEp24 sequence flanking the MCS was used to define the ends of the insert fragment.

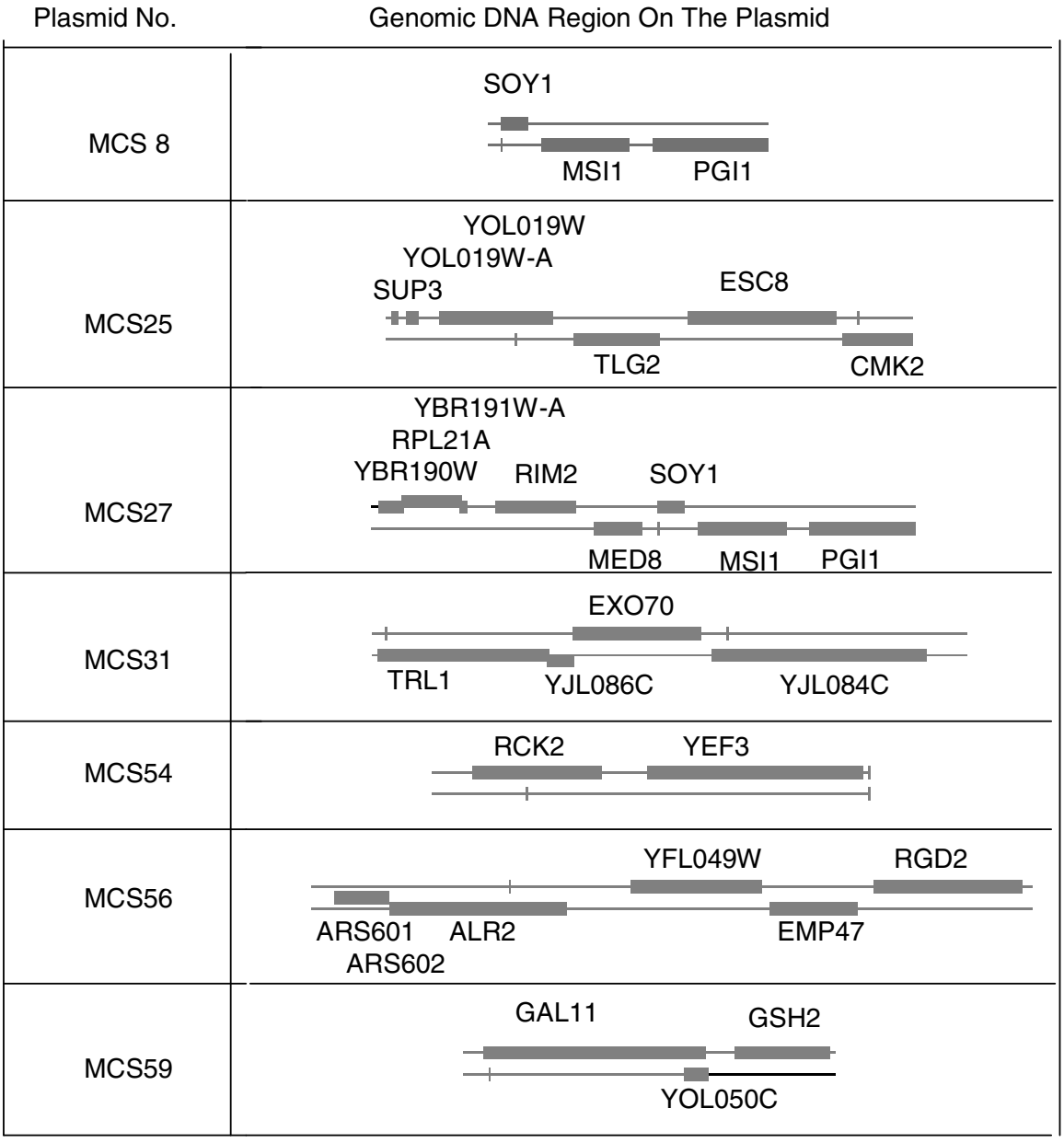
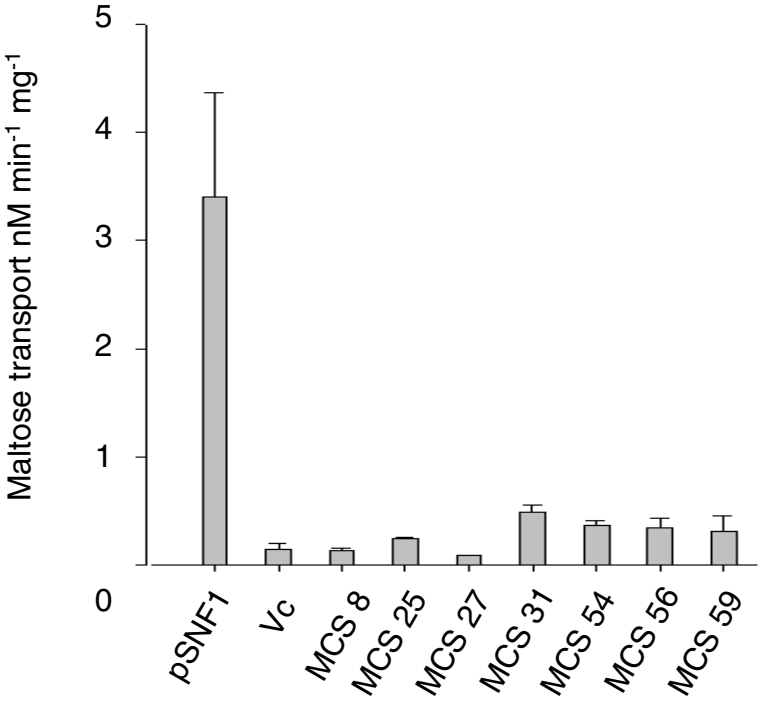


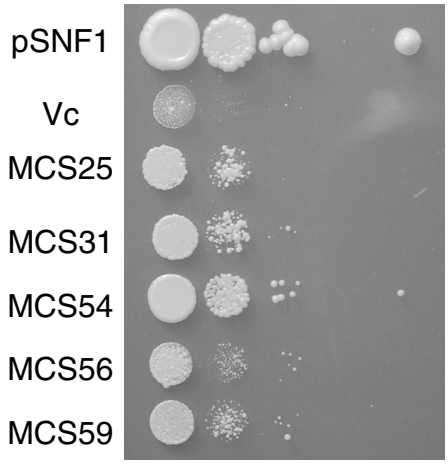
Figure 25. Four out of seven suppressor plasmids allow for increased maltose transport activity in the *snf1*Δ *mig1*Δ [pMAL63/43-c]

CMY101 (*snf1*Δ *mig1*Δ) was transformed with pUN30/MAL63/43-c and one of the multicopy suppressor plasmids, pCE9 (YEp24/SNF1) or YEp24 (Vc). Cells were grown in 0.1% glucose, washed with ddH2O, and resuspended in selective medium containing 2% maltose. Cultures were returned to the 30^{o} C incubator and allowed to grow for another six hours. Transport assays were performed as described in Material and Methods and are shown in Panel A. Panel B shows the growth rate of CMY101 pMAL63/43-c transformants carrying various multicopy suppressors as indicated. For this, the transformants were grown in selective synthetic medium with 0.5% glucose; cultures were diluted to a density of 10^{7} cells per mL; and serial dilutions (1/10) were spotted (5 µL) onto agar medium containing 2% maltose and same selective nutrients. These were photographed after incubation for two days at 30^{o} C and two days at room temperature.

A.


Maltose transport nM min-1 mg-1
5
4
3
2
1
0
pSNF1
Vc
MCS 8
MCS 25
MCS 27
MCS 31
MCS 54
MCS 56
MCS 59

B.


pSNF1
Vc
MCS25
MCS31
MCS54
MCS56
MCS59

c] transformants carrying the multicopy suppressors grow poorly compared to the *SNF1* control. Out of the five multicopy suppressor plasmids, I decided to characterize MCS31, 54, 56 and 59 due to their better growth on 2% maltose and the enhanced maltose transport activity displayed by them.

Characterization of multicopy suppressor 54 reveals a serine/threonine kinase encoded by RCK2 as the suppressor of the snf1Δ mig1Δ Mal⁻ phenotype

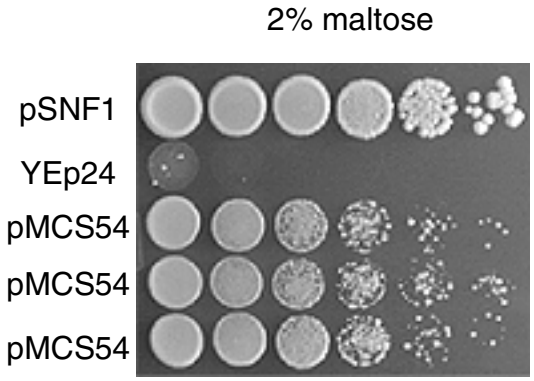
The plasmid designated MCS54 was recovered from the independent *snf1Δ mig1Δ* [pMAL63/43-c] transformant. The sequence analysis on MCS54 revealed a region of *S. cerevisiae* chromosome XII. The approximately 6.5kb insert fragment contained two open reading frames *RCK2* and *YEF3* (Figure 24). *RCK2* encodes a serine/threonine kinase whereas *YEF3* codes for yeast elongation factor 3 (Dahlkvist et al., 1995; Qin et al., 1987). Both of these genes were individually subcloned into the multiple cloning site of YEp24 by amplifying the genomic DNA fragment containing single gene sequences using MCS54 as template.

YEp24 plasmids containing single gene insert were transformed into *snf1Δ mig1Δ* [pMAL63/43-c] and maltose transport activity determined. Cultures were grown in minimal medium lacking uracil and tryptophan plus 0.1% glucose before being transferred to same selective medium and 2% maltose. Transformants carrying *RCK2* in multiple copies per cell showed increased maltose transport compared to the vector control (Figure 26B). *YEF3* transformants showed no

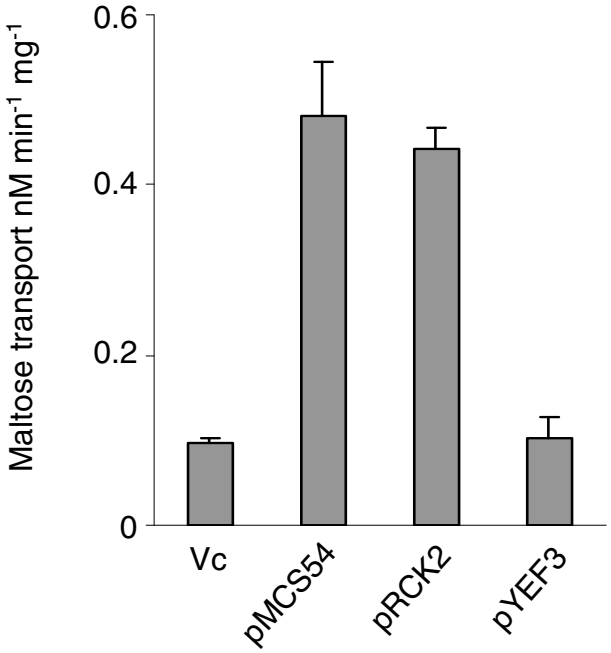
Figure 26. *RCK2* improves maltose transport in *snf1Δ mig1Δ* [pMAL63/43-c]

Growth on 2% maltose was compared for CMY101 (*snf1*Δ *mig1*Δ) [pMAL63/43-c] strain carrying YEp/SNF1, YEp24 (Vc), or three independent pMCS54 transformants by dilution assay (Panel A). As described in Figure25, serial dilutions (1/5) were spotted (7.5 µL) onto agar plates containing 2% maltose. Panel B: Strain CMY101 carrying the constitutive *MAL*-activator was transformed with one of the following: YEp24 (Vc), pMCS54, YEp24/RCK2, and YEp24/YEF3. Cultures were grown as described in Figure 25. Maltose transport activities were determined as described in Materials and Methods.

A.


2% maltose
pSNF1
YEp24
pMCS54
pMCS54
pMCS54

B.


Maltose transport nM min-1 mg-1
0.6
0.4
0.2
0
Vc
pMCS54
pRCK2
pYEF3

increase in transport activity. This identifies *RCK2* as the multicopy suppressor in MCS54. *RCK2* encodes a serine/threonine protein kinase (Dahlkvist et al., 1995).

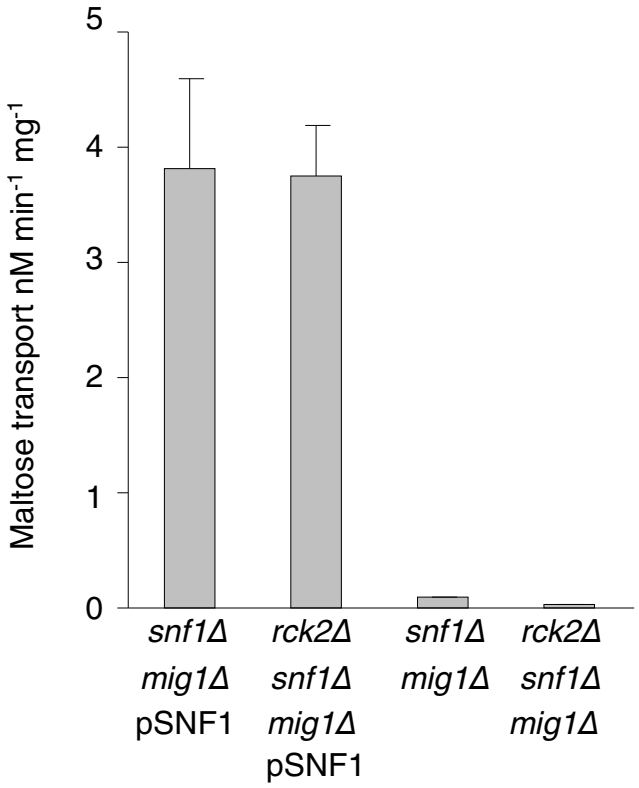
In an effort to reveal the role of Rck2 kinase in maltose permease expression/trafficking, I decided to evaluate an *rck2Δ* strain. Previous studies showed that strains carrying a deletion of *RCK2* are viable (Dahlkvist et al., 1995). I constructed the *snf1Δ mig1Δ rck2Δ* triply deleted strain and tested its maltose utilization phenotype. The genomic copy of *RCK2* was replaced with the selectable marker gene *Kanamycin*^{R} in *snf1Δ mig1Δ* creating a triple deletion *snf1Δ mig1Δ rck2Δ*. Both of these strains were transformed with pMAL63/43-c carrying a constitutive *MAL*-activator and pCE9 carrying *SNF1* in YEp24 and maltose transport activities determined. Both strains showed transport activities typical of the controls (Figure 27). Thus no new phenotype was identified. Results suggest that Rck2 kinase is not generally required for maltose permease expression and maltose utilization in presence or absence of *SNF1*. This also means that overexpression of Rck2 kinase bypasses the need for Snf1 protein kinase, and Snf1p and Rck2p are not components of the same or parallel metabolic or regulatory pathways regulating maltose transport activity.

A component of the Mediator complex is identified as a suppressor of the maltose non-fermenting phenotype of snf1Δ mig1Δ [pMAL63/43-c]

The sequencing analysis of the vector junction region of MCS59 found that it contained a region of chromosome XV. The roughly 5.8kb piece carried

Figure 27. *RCK2* is not required for maltose utilization

Strains CMY101 (*snf1*Δ *mig1*Δ) and CMY 115 (*snf1*Δ *mig1*Δ *rck2Δ*) carrying pUN30/MAL63/43-c were transformed with either YEp24/SNF1 (A, B) or YEp24 (C, D). Cultures were grown in selective synthetic medium lacking uracil and tryptophan and 0.1% glucose medium. Cells were harvested by filtration, resuspended in the same selective medium containing 2% maltose, and allowed to grow for another six hours before assays were performed. Maltose transport activities were assayed for three individual transformants as described in Materials and Methods.


Maltose transport nM min-1 mg-1
5
4
3
2
1
0
snf1Δ mig1Δ pSNF1
rck2Δ snf1Δ mig1Δ pSNF1
snf1Δ mig1Δ
rck2Δ snf1Δ mig1Δ

two genes *GAL11* and *GSH2*. Gal11p is a component of the yeast mediator complex and regulates transcription of a variety of genes including the *MAL*-structural genes by acting as a target of transcription activators and repressors (Bjorklund and Gustafsson, 2005; Wang and Michels, 2004). Gsh2p, glutathione synthetase, catalyzes the ATP-dependent synthesis of GSH from gamma-glutamylcysteine (gamma-Glu-Cys) and glycine (Inoue et al., 1998).

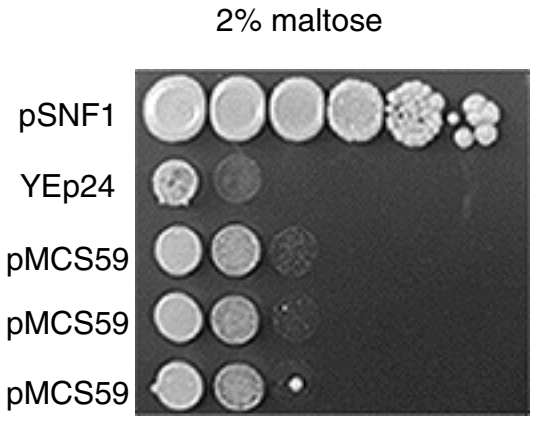
Both *GAL11* and *GSH2* were amplified by PCR using the MCS59 plasmid as template. The resulting genomic fragments were inserted into the multiple cloning site of YEp24 and transformed into *snf1Δ mig1Δ* [pMAL63/43-c]. Transformants of each were grown in selective synthetic medium containing 0.1% glucose before transferring to same selective medium containing 2% maltose for six hours. The *snf1Δ mig1Δ* [pMAL63/43-c] strain transformed with pGAL11 showed increased maltose transport activity compared to transformants carrying vector alone (Figure 28). *GSH2* transformants exhibited no increase in maltose transport compared to the negative control. This indicates that *GAL11* is the suppressor gene of MCS59.

Extensive studies on mediator complex have revealed the role of Gal11p in transcription and *gal11Δ* mutant strains are viable (Giaever et al., 2002). Wang and Michels (2004) showed that deletion of *GAL11* significantly decreases the induced and basal level expression of maltase. Since the *gal11Δ* strain was shown to be defective for maltose induction even in presence of Snf1 kinase, it suggests the Gal11p as the positive regulator of *MAL* gene transcription rather than at the post-transcriptional level. This is addressed below.

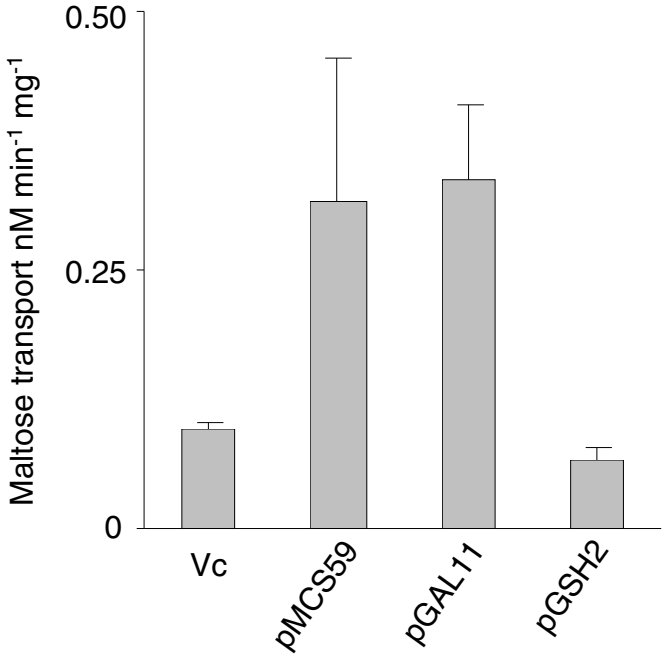
Figure 28. Suppression of growth defects of *snf1Δ mig1Δ* [pMAL63/43-c] by multicopy *GAL11* gene

Strain CMY101 (*snf1*Δ *mig1*Δ) [pMAL63/43-c] was transformed with one of the following: YEp24/SNF1, YEp24 or pMCS59. Serial dilution assay was performed as described in Figure 26 (Panel A). The same doubly deleted strain carrying the constitutive *MAL*-activator was transformed with one of the following: YEp24 (Vc), pMCS59, pGAL11, or pGSH2. Cultures were grown as described in Figure 25. Maltose transport activities were assayed as described in the Materials and Methods section (Panel B).

A.


2% maltose
pSNF1
YEp24
pMCS59
pMCS59
pMCS59

B.


0.50
0.25
0
Maltose transport nM min-1 mg-1
Vc
pMCS59
pGAL11
pGSH2

Characterization of MCS31 and MCS56

The sequencing of vector junction regions of MCS31 and MCS56 revealed portions of chromosomes X and VI, respectively (Figure 24). MCS31 contains a roughly 9 kb insert whereas MCS56 carries an approximate 10 kb piece. MCS31 contains four open reading frames including *EXO70*, *TRL1*, *YJL084C* and *YJL086C*, a hypothetical gene. MCS56 also carries four open reading frames namely *ALR2*, *EMP47*, *RGD2* and *YFL049W*, a gene of unknown function. All genes present on both suppressor plasmids were individually subcloned into YEp24 vector and tested for their ability to restore maltose transport in the *snf1Δ mig1Δ* [pMAL63/43-c] strain. None however, individually allowed for increased maltose transport activity. Analysis of genomic insert sequence did not reveal any previously unknown open reading frames. Further efforts to determine if two or more of the genes carried by MCS31 or MCS56 were required for suppression were unproductive.

Gal11p suppresses snf1Δ mig1Δ [pMAL63/43-c] defects at the level of transcription

In an effort to localize the point in maltose permease expression at which the suppressor genes were involved, I decided to look at the Mal61p/HA levels in *snf1Δ mig1Δ* [pGPD-MAL61/HA] strain in which permease gene transcription is completely independent of the native promoter.

The plasmid carrying the isolated suppressor genes *RCK2* and *GAL11* were transformed into the *snf1Δ mig1Δ* strain along with pGPD-MAL61/HA.

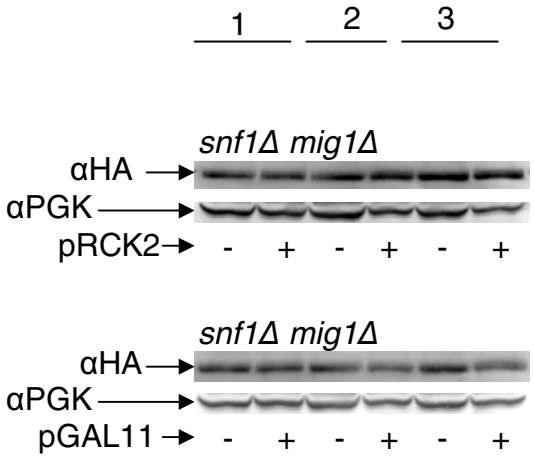
Individual transformants of each were grown in selective minimal medium containing 0.1% glucose. Their respective maltose transport activities were determined along with the Western blot analysis of Mal61p/HA. The Western blot results reported in Figure 29A suggest that overexpression of neither *RCK2* nor *GAL11* enhances the Mal61 protein levels. Transformants carrying pRCK2, however, displayed increased maltose transport activity compared to the vector control while those carrying pGAL11 did not (Figure 29B). This result confirms the involvement of *GAL11* at the level of transcription since in the absence of the *MAL61* promoter, multicopy *GAL11* fails to suppress the *snf1Δ mig1Δ* Mal$^-$ phenotype. Although overexpression of *RCK2* does not increase Mal61p levels, it enhances maltose transport. This suggests that it may allow the escape of maltose permease to the plasma membrane and/or activate the maltose transport of the permease.

Even though I was unable to identify the suppressor genes on both MCS31 and MCS56, I decided to evaluate the step in Mal61p expression at which these suppressor plasmids acted. I transformed *snf1Δ mig1Δ* [pGPD-MAL61/HA] strain with the two suppressor plasmids and the resulting transformants were grown in 0.1% glucose. Western blot results reported in Figure 30A show no significant improvement in Mal61p/HA levels compared to the control. Nevertheless, in presence of either of the MCS plasmids, the *snf1Δ mig1Δ* [pMAL63/43-c]strain exhibited increased maltose transport activity (Figure 30B). These results imply that both MCS31 and MCS56 are involved in permease activation and/or trafficking.

Figure 29. *RCK2* and *GAL11* do not increase Mal61p levels in the absence of *SNF1*

CMY101 (*snf1*Δ *mig1*Δ) was transformed with p423/GPD-MAL61/HA and either YEp24/RCK2 or YEp24/GAL11. Three individual transformants of each were grown in minimal medium lacking histidine and uracil and 0.1% glucose. Cells were collected by filtration, and used for Western blot analysis as described in Material and Methods (Panel A). Panel B shows the maltose transport activities of transformants of CMY101 [pGPD-MAL61/HA] carrying: YEp24 (Vc), YEp24/RCK2, and YEp24/GAL11. Maltose transport was determined as described in Materials and Methods section.

A.


1
2
3
snf1Δ mig1Δ
αHA
αPGK
pRCK2
- + - + - +
snf1Δ mig1Δ
αHA
αPGK
pGAL11
- + - + - +

B.

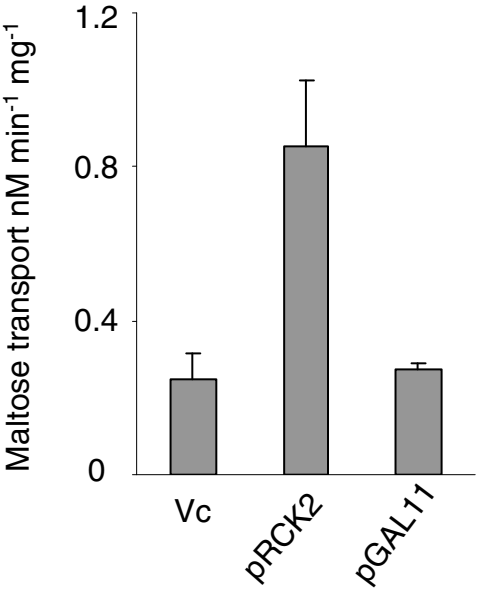
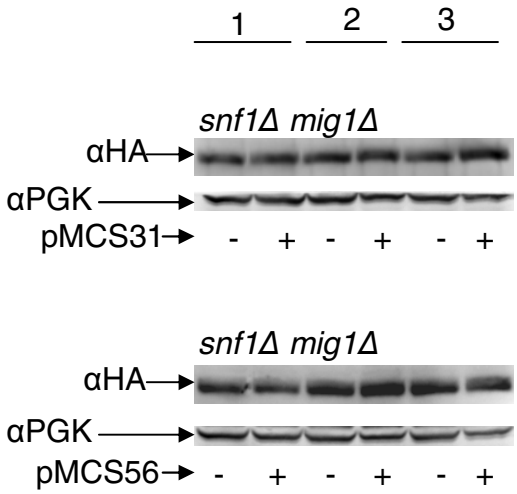

Maltose transport nM min-1 mg-1
1.2
0.8
0.4
0
Vc
pRCK2
pGAL11

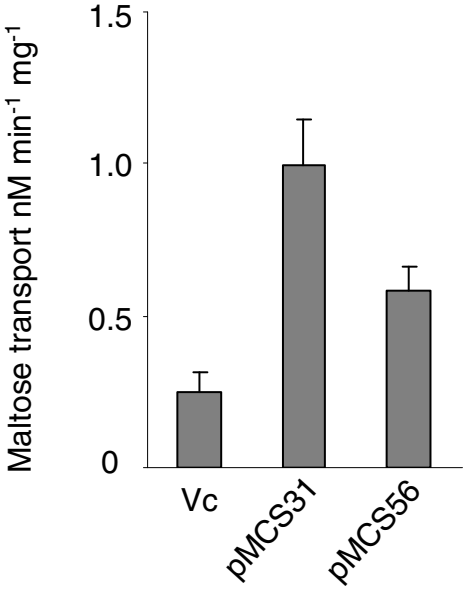
Figure 30. The Multicopy suppressor plasmids 31 and 56 act at the post-translational level in *MAL61* expression in *snf1Δ mig1Δ* strain

CMY101 (*snf1*Δ *mig1*Δ) carrying p423/GPD-MAL61/HA was transformed with either pMCS31 or pMCS56. Three individual transformants of each were grown to mid log phase as described in Figure 29. Two sets of samples were collected to be used for Western blot analysis (Panel A) and maltose transport assay (Panel B) as described in Materials and Methods section.

A.


1
2
3
snf1Δ mig1Δ
αHA
αPGK
pMCS31
- + - + - +
snf1Δ mig1Δ
αHA
αPGK
pMCS56
- + - + - +

B.


Maltose transport nM min-1 mg-1
1.5
1.0
0.5
0
Vc
pMCS31
pMCS56

DISCUSSION

In this chapter, I report the identification of two genes, *RCK2* and *GAL11* that act as weak multicopy suppressors of the maltose growth defects of a *snf1Δ mig1Δ* [pMAL63/43-c] mutant. Increased dosage of *RCK2* or *GAL11* only partially compensates for defects caused by the *snf1Δ* by improving maltose uptake but do not enhance maltose permease protein levels. In addition I report the isolation of two regions of the *S. cerevisiae* genome, which when present in high copy, result in improved maltose transport in a *snf1Δ* strain.

RCK2 appears to enhance activation and/or trafficking aspect of maltose permease but not protein levels

S. cerevisiae RCK2 and its homologue *RCK1* were identified as suppressors of a cell cycle check point mutation in *S. pombe* (Dahlkvist et al., 1995). Rck2p, a serine/threonine kinase, is homologous to mammalian calmodulin dependent kinase (Teige et al., 2001). It is involved in inhibiting meiosis in *Saccharomyces* as *RCK2* mutant diploids enter meiosis with a high efficiency and more rapidly than wildtype (Ramne et al., 2000).

A better understanding of *RCK2* function comes from Bilsland-Marchesan et al., (2000), who described it as substrate of Hog1 kinase. In presence of high osmolarity stress, the MAPK Hog1p induces a set of survival responses. Rck2p is a substrate for Hog1p as its phosphorylation by Hog1p results in increased kinase activity (Bilsland-Marchesan et al., 2000). Teige et al., (2001) demonstrated that Hog1p dependent activation of Rck2p directs it to

phosphorylate translation elongation factor 2. This post-transcriptional regulation response to stress, results in attenuation of protein synthesis. Since stress causes inhibition of translational rate and Rck2p is shown to inhibit translation EF-2, this suggests a role for Rck2p as a negative regulator of translation. Stress activated MAP kinase pathways are implicated in regulating stability and the loading of mRNAs onto ribosome (Swaminathan et al., 2006). Roles of Rck2p in negative regulation of translation elongation include: 1) slow translation of an mRNA once the first ribosome has loaded, 2) increased accumulation of stalled polysomes, and 3) increased stalling promotes translation of mRNA with weak binding affinity to initiation factors at an expense of mRNAs with stronger binding properties (Swaminathan et al., 2006).

In my search for suppressors of *snf1* mutant phenotype, I isolated *RCK2* as a multicopy suppressor of maltose metabolism defects. My results suggest that Rck2 kinase is not normally involved in regulating maltose permease. Deletion of *RCK2* does not cause a maltose utilization defect or affect maltose transport activity (Figure 27). Thus *RCK2* is not a downstream component of a Snf1 kinase pathway regulating maltose permease synthesis or activation. Nor is it a component of a parallel pathway regulating maltose permease. Instead, I suggest that multicopy *RCK2* is a by-pass-suppressor of *snf1Δ* and when over produced, is capable of mimicking the role of Snf1 kinase in permease synthesis or activation. Wang et al., (2001) had also isolated *RCK2* as a suppressor of a glycogen deficient phenotype of *snf1Δ pcl8Δ pcl10Δ*. Similar to our results they

reported that absence of *RCK2* does not enhance the glycogen deficient phenotype.

In view of the role of Rck2 kinase as a negative regulator of translation, it is somewhat of a surprise to isolate *RCK2* as a multicopy suppressor of *snf1Δ*. Notably, Mal61p/HA levels do not change significantly in response to high-copy *RCK2* but transport activity is increased (Figure 29) suggesting that the mechanism of *RCK2* suppression does not involve translational regulation but involves activation of the transport function. Interestingly, Zrc1, the Zn^{+} transporter, was found to be a direct target of Rck2 kinase for they co-immunoprecipitate (Bilsland et al., 2004).

Perhaps overproduction of Rck2 kinase enables it to act directly on maltose permease and thereby activate transport albeit poorly. If this is correct, it could suggest that Snf1 kinase acts directly on maltose permease to regulate its transport activity and perhaps also stability and trafficking.

GAL11 suppresses the snf1 mutation defects at the transcriptional level

GAL11 encodes a transcription factor that is a component of the SRB/Mediator sub-complex of RNA polymerase II holoenzyme. *GAL11* is required for full expression of many genes of *S. cerevisiae* (Badi and Barberis, 2001; Sakurai et al., 1994). It functions either positively or negatively, depending on the structure of the target promoters and combination of DNA-bound activators (Suzuki and Nishizawa, 1994).

In maltose metabolism, Gal11p plays a significant role in *MAL* gene

transcription. A strain lacking *GAL11* displays much reduced levels of maltase activity almost at non-induced levels (Wang and Michels, 2004). This suggests that Gal11p has a positive function in maltose metabolism and *MAL* gene transcription. Snf1 kinase, which is generally shown to be involved at the level of transcription by phosphorylation of activators and repressors, has also been shown to interact with the components of the SRB/Mediator complex (Kuchin et al., 2000). Therefore it is not surprising that overexpression of a member of the RNA polymerase machinery, which is also a known as a player in *MAL* gene transcription, bypasses the need for Snf1 kinase but only when permease is expressed from its native promoter which is *GAL11* dependent. When expressed from the constitutive GPD promoter increased maltose transport is not observed (Figure 29). The modest suppression by multicopy *GAL11* found in the *snf1Δ mig1Δ* [pMAL63/43-c] is likely to result from a modest increase in permease protein that is not readily detected.

Potential suppressor genes of MCS31 and MCS56

In addition to isolation of *RCK2* and *GAL11* as multicopy suppressors, I also isolated two plasmids (MCS31 and MCS56) carrying genomic regions of *S. cerevisiae* chromosome X and VI (Figure 24). I was not able to identify the suppressor genes in these plasmids. Since neither MCS31 nor MCS56 increased permease protein levels, it is likely that improved trafficking to the plasma membrane or activation may be the mechanism of suppression. For this reason I suspect that *EXO70* and *EMP47* are the likely candidates for the

suppressor genes. Emp47p is an integral membrane component of endoplasmic reticulum-derived COPII-coated vesicles, which function in ER to Golgi transport (Sato and Nakano, 2002). It is shown to function in glycoprotein secretion. It is possible that overexpression of *EMP47* bypasses the need for Snf1p by enhancing trafficking of Mal61p between ER and Golgi.

EXO70 is the essential component of the exocyst complex which acts to mediate polarized targeting of secretory vesicles to active sites of exocytosis (TerBush et al., 1996). In support of this hypothesis overexpression of Snf1 kinase has been shown to rescue the secretory defective phenotypes associated with *sec10* and *sec15* mutant strains both of which are components of the exocyst complex (Elbert et al., 2005). Because maltose permease protein levels are so low in the *snf1Δ* strain, I have been unable to explore this possibility.

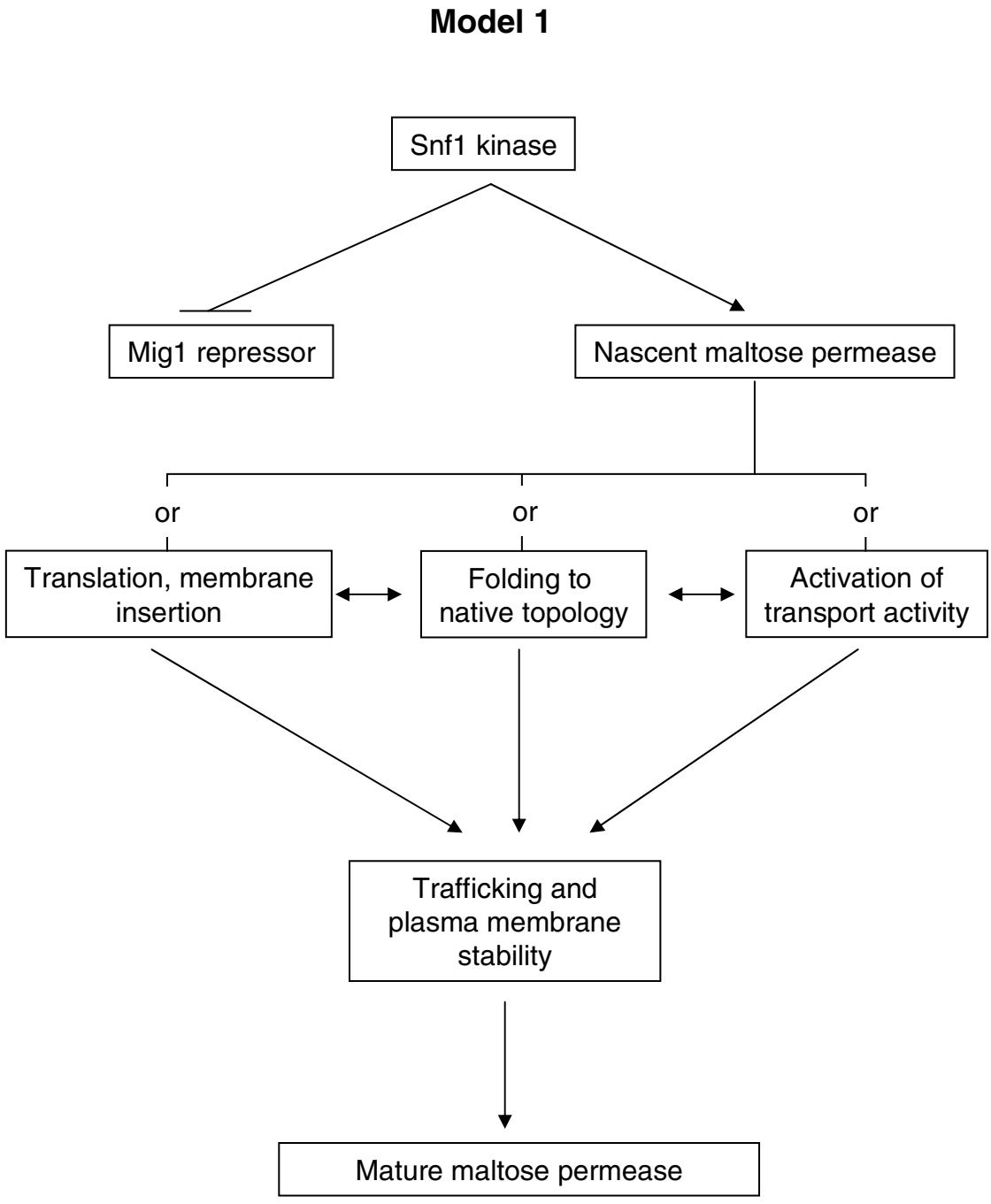
CONCLUSIONS

The major finding of my thesis is that Snf1 kinase is essential for the synthesis of active, plasma membrane localized maltose permease. The role of Snf1 kinase as a regulator of transcription factors, such as Mig1 repressor, is well established (Carlson, 1999; Johnston, 1999). Here, I demonstrate a novel post-transcriptional function for Snf1 kinase in the expression of an integral membrane protein.

I demonstrate that loss of Snf1 kinase leads to a dramatic decrease in the level of maltose permease protein synthesized despite nearly normal levels of mRNA. One important cause of this low level of maltose permease protein is my finding that, in the absence of *SNF1*, maltose permease protein is a target of the ER/Golgi quality control enzymes. In addition, I showed that the very low protein levels present in a *snf1Δ* mutant is functionally inactive and rapidly internalized from the plasma membrane. The total impact of these pleiotropic effects of *snf1Δ* is a severe defect in maltose transport activity and the noninducible phenotype of a *snf1Δ mig1Δ* [pMAL63/43-c] strain observed by Hu et al. (2000). This function of Snf1 kinase is novel. (Dong and Dickson, 1997; Wiedemuth and Breunig, 2005) report that *K. lactis* Lac4p and Lac12 permease expression is defective but the underlying mechanism of this defect is not explored.

I propose two models to explain how Snf1 kinase causes these pleiotropic defects. Model 1 (Figure 31) suggests that Snf1 kinase, directly or indirectly, activates a single essential function uniquely required for maltose permease synthesis or activation. These include its translation and membrane insertion; its

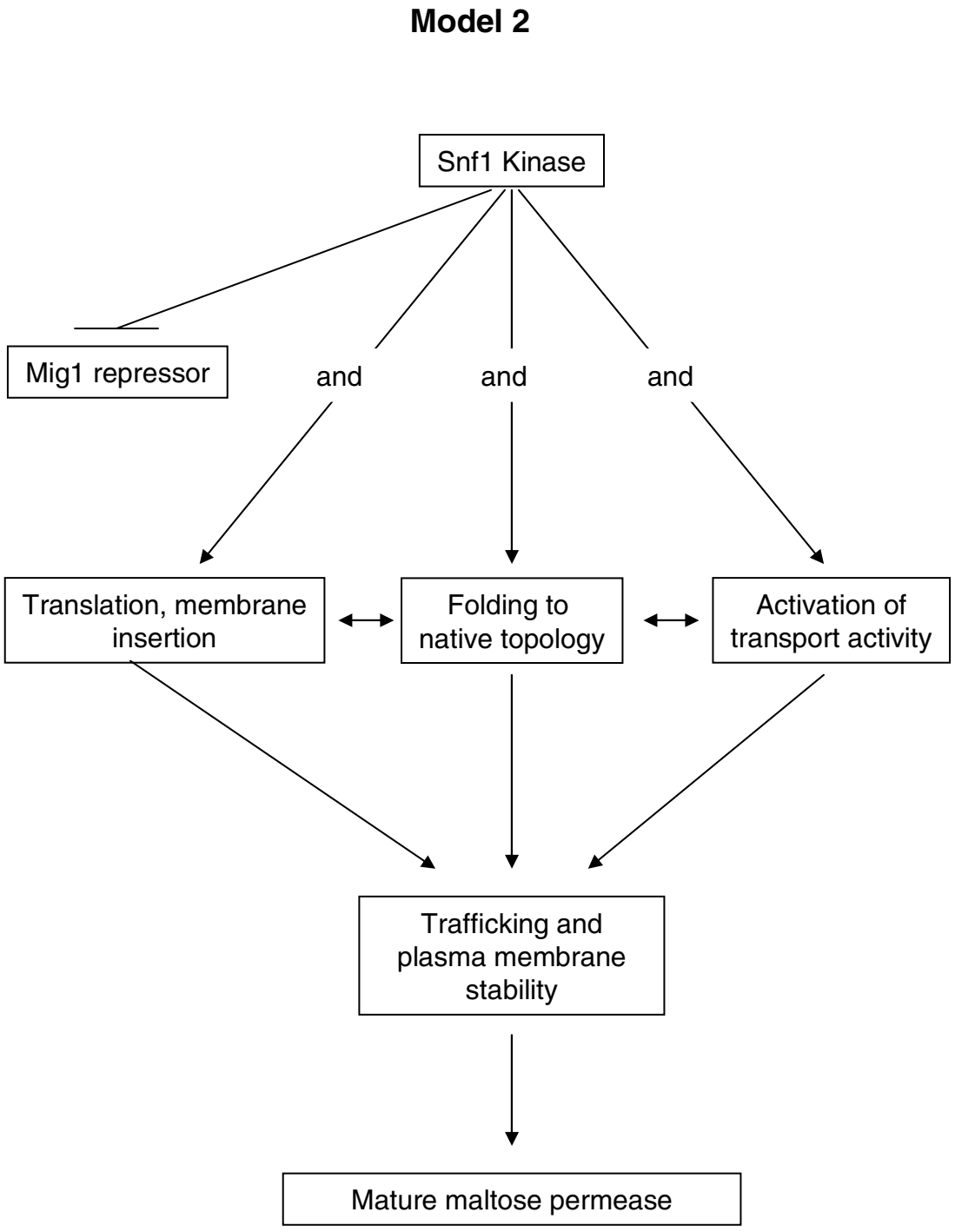
Figure 31. Proposed model 1 illustrating the Snf1 protein kinase dependency of maltose permease expression in *snf1Δ mig1Δ* strain



folding to the active topology, or the activation of transport activity. Examples of how these might be regulated are the following. Perhaps phosphorylation of maltose permease is required for activity and folding and Snf1 kinase might carry this out directly or via the activation of another kinase. Gadura et al. (2006) showed that *YCK1* and *YCK2* mutant strains, which express temperature sensitive Yck1, Yck2 casein kinase, express normal levels of plasma membrane localized maltose permease but that this permease is functionally inactive. Alternately, Snf1 kinase could activate a chaperone required for folding and/or membrane insertion of maltose permease. Gsf2p is an example of such a chaperone and is required for Hxt1p and Gal2p expression (Sherwood and Carlson, 1999). Shr3p is an ER packaging chaperone, required for incorporation of amino acid permeases into COPII coated vesicles for transport to the cell surface and has been shown to interact with maltose permease (Gilstring et al., 1999; Miller et al., 2005). As is suggested in Model 1, if these functions are achieved properly, the nascent maltose permease is able to move to the plasma membrane in an active form and be retained at the plasma membrane. Model 2 (Figure 32) proposes that Snf1 kinase acts, directly or indirectly, at several steps in the synthesis of maltose permease each of which is required for normal expression of active plasma membrane localized maltose permease.

Unfortunately, the multicopy suppressor analysis undertaken in Chapter 3 did not reveal a greater understanding of the mechanism of this Snf1 kinase requirement in maltose permease synthesis/maturation. It was hoped that this would identify a downstream target of Snf1 kinase such as a maltose permease

Figure 32. Proposed model 2 for the Snf1 protein kinase dependency of maltose permease expression in *snf1Δ mig1Δ* strain



specific chaperone, kinase, or other such modification enzyme. The possibility remains that Snf1 kinase acts directly on the nascent maltose permease and/or that the requirement for Snf1 kinase is so specific that other kinases, even when overexpressed, are unable to adequately accomplish this function. Nonetheless, we prefer Model 1 because it provides the simplest and most straightforward explanation.

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