Msi1p accumulation is dependent on carbon stress and cold stress, but is not controlled by ubiquitin tagging

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The Msi1 protein (Msi1p) is a *Saccharomyces cerevisiae* protein known to be involved in suppression of the RAS pathway, and folding of newly replicated DNA around histones H3 and H4 in the CAF-1 complex. Furthermore, its human orthologs, RbAp46 and RbAp48 are hypothesized co-repressors of transcription with the retinoblastoma protein (pRB). We have identified a fourth function of Msi1p, in which Msi1p activates transcription in a two-hybrid system without a prey protein. This condition, which we term the one-hybrid activity of Msi1p suggests Msi1p can act as a transcription factor in *S. cerevisiae*. More interestingly, this one-hybrid activation occurs on nonfermentable carbon sources, but not on fermentable carbon sources. Western blot analysis of Msi1p in fermentable and non-fermentable carbon sources has indicated the one-hybrid activation may be due to increased accumulation of Msi1p in non-fermentable carbon sources. It is believed this increase of Msi1p may be due to the carbon source stress of aerobic growth compared to the preferred anaerobic growth of *Saccharomyces cerevisiae*. Msi1p also accumulates in both dextrose and glycerol during cold stress. We hypothesize activation of Msi1p in these two stresses occur independently of one another.

**Introduction**

*Saccharomyces cerevisiae* is used as a model organism for study because, like a mammalian cell, *S. cerevisiae* is eukaryotic. Though *S. cerevisiae* is unicellular, many of the cellular pathways that occur in yeast are homologous to mammalian cellular pathways. Thus, *S. cerevisiae* can provide a useful model for study of mechanisms that are present in mammalian cells. The ability and success of using *S. cerevisiae* as a model organism depends largely in part on the high conservation of transcriptional and translational machinery that is shared between yeast and mammalian cells. The advantages of using *S. cerevisiae* as a model organism include the speed at which *S. cerevisiae* can generate new daughter cells. *S. cerevisiae* can continue to generate new cells for multiple generations and, unlike mammalian cells that require genetic mutations in order to be immortalized, cultures of *S. cerevisiae* can continue for a long period of time with adequate nutrients. The genome of *S. cerevisiae* is well understood in
comparison to many higher eukaryotes, which allows for better understanding of cellular pathways. Though *S. cerevisiae* are complex organisms, their simplicity compared to mammalian cells provides a more efficient model to study genetics, signal transduction pathways, aging, and telomere shortening. Finally, *S. cervisiae* can be transformed—or mutated—easily by addition of plasmid DNA and deletion of chromosomal DNA by recombination.

For the past three years, the goal of the North Central College Yeast Lab has been to understand the molecular mechanisms that govern expression of the Msi1 protein (Msi1p) in the yeast *S. cerevisiae*. Msi1p is known to be involved in three separate pathways within *S. cerevisiae*. The RAS/cAMP signal transduction pathway in *S. cerevisiae* mediates nutrient response. As RAS, a known human oncogene, promotes cAMP production by activating adenylate cyclase, the cell grows, which in return limits expression of heat shock proteins (HSPs). Activation of cAMP leads to glycogen utilization, increased glycolysis, activation of growth-related genes, and sensitivity to transient heat shock (Broach and Dechenes, 1990). Overactive RAS/cAMP pathway mutants increase cAMP levels in *S. cerevisiae* due to constitutive activation of RAS and the pathway. HSPs are chaperone proteins important in protecting cellular proteins from misfolding during environmental stress, including heat shock. This protection during environmental stress leads to a loss of growth and increased accumulation of trehalose, a disaccharide. Overactive RAS/cAMP will lead to sensitivity during heat stress because cells are not protected from the heat. The result is less trehalose accumulation, and reduced HSP expression, which are synthesized to help fold denatured proteins.
In the late 1980s, Msi1p was identified as a protein involved in suppressing the heat shock sensitive phenotype typical of the activated RAS/cAMP pathway in S. cerevisiae (Ruggieri et al., 1989). Loss of msi1 in S. cerevisiae with an overactive RAS/cAMP pathway are heat shock sensitive (Ruggieri et al., 1989). Overexpression of Msi1p in activated RAS/cAMP pathway mutants show decreased cAMP levels and silencing of the constitutive activity of the mutant activated pathway (Ruggieri et al., 1989). Effectively, suppression of the overactive RAS/cAMP pathway by Msi1p rescues the heat-sensitive phenotype through reduction of the cAMP levels within S. cerevisiae allowing expression of HSPs, and for adequate resistance to heat shock. Though Msi1p is necessary for suppression of the RAS/cAMP pathway, MSL1 is not essential for growth of S. cerevisiae (Ruggieri et al., 1989). Msi1p does not seem to have a direct effect on cAMP levels though. Rather, suppression of the activated RAS/cAMP pathway mutation depends on interaction between Msi1p and Npr1p (nitrogen permease reactivator-1) (Johnston et al., 2001).

In 1997, the chromatin assembly complex 3 (CAC3) gene was identified as part of a chromatin assembly factor 1 (CAF-1) that helps fold newly replicated DNA about histones H3 and H4 (Kaufman et al., 1997). To the surprise of the laboratory studying the CAC3 gene, the genomic coding sequence was identical to MSL1 (Kaufman et al., 1997). Loss of any one of the three CAC genes causes chromatin defects, changing the expression of several genes (Kaufman et al., 1997). But the loss of either CAC1 or CAC2 does not have any effect on the ability of the Msi1p to suppress the RAS/cAMP pathway (Kaufman et al., 1998). Loss of any CAC gene does not lead to cell death, indicating chromatin folding in S. cerevisiae must have other processes to control folding (Johnston et al., 2001).
et al., 2001). But, loss of any CAC gene leads to an increase in UV radiation sensitivity (Kaufman et al., 1997).

The retinoblastoma protein (pRB) is a tumor suppressor that is required for repression of some genes. pRB is important in the history of cancer study because it was the subject of Alfred Knudson’s proposed two-hit model of cancer. This model, which is applied for most tumor suppressor genes, notes both copies of a tumor suppressor gene must be mutated in order for a loss of the tumor suppressor function (Russell, 2002).

During the cell cycle, pRB controls the G1 to S check point. Unphosphorylated pRB binds to the DP1 and E2F transcription factors, inhibiting transcription of genes controlled by E2F (Russell, 2002). This keeps the cell in a growing or resting state. During the transition to S phase where DNA is duplicated, pRB is phosphorylated by a cyclin/cyclin dependent kinase (Cdk) complex (Russell, 2002). The phosphorylated pRB releases the DP1 and E2F transcription factors, allowing transcription of genes controlled by E2F (Russell, 2002). This results in translation of proteins needed for entry into the S phase of the cell cycle.

When both copies of the RB gene are mutated, pRB cannot bind to DP1 and E2F, allowing transcription of E2F genes (Russell, 2002). This leads to a loss in the G1 to S phase regulation. Cells are able to transition from G1 to S phase without correcting DNA damage. Uncorrected DNA damage can lead to genetic mutations that will remain in cell lines once mitosis has been completed.

When the mammalian tumor suppressor, pRB, is transformed into S. cerevisiae, MSI1 is required for repression of transcription (Kennedy et al., 2001). Studies in S. cerevisiae have shown MSI1 is required for exogenous pRB to repress genes.
Furthermore, MSII shares homology with the human genes, RbAp46 and RbAp48, hypothesized to recruit histone deacetylases (HDACs) for pRB (Kennedy et al., 2001). Histone deacetylases are a family of proteins found in all eukaryotes and involved in silencing gene expression. DNA is wrapped around histones to package the DNA into the nucleus of an eukaryotic cell. HDACs repress transcription and gene expression by removing polar uncharged acetyl groups from histones leaving a positively charged lysine residue. The removal of acetyl groups causes the histones to bind more tightly to the anionic DNA, prohibiting the DNA from being transcribed by the RNA polymerases. Both RbAp proteins have been shown to co-purify with histone deacetylases (Taunton, 1996), as well as bind to pRB (Qian, 1995). One hypothesized function of these orthologs is to bind to pRB, which recruits HDACs to pRB, repressing transcription of pRB transcripts (Kennedy et al., 2001). It is not known whether RbAp46 and RbAp48 are also involved in chromatin assembly.

MSII is involved in establishing chromatin structure, whether Msi1p is to fold newly synthesized DNA, or to possibly repress transcription of genes. One hypothesis is Msi1p could recruit HDACs in tightening the binding of histone proteins to the DNA, which would silence gene expression. MSII has been found to be expressed continually throughout the cell cycle, whereas CAC1 and CAC2 mRNA expression are regulated, peaking during G1 phase (Spellman et al., 1998). This suggests the function of Msi1p as a repressor of transcription or chromatin folder is dependent on which protein(s) Msi1p binds.

One Hybrid activity of MSII
Msi1p has been found to have a fourth function. In two-hybrid assays, a researcher asks whether two proteins interact with each other. In *S. cerevisiae* two-hybrid screens, the DNA binding domain (DBD) of Gal4 is fused to a bait protein, and the Gal4 activation domain (AD) is fused to a prey protein. When the proteins interact with each other, they activate transcription of a marker gene that encodes a protein that can synthesize a specific reporter gene. The interaction is assayed by the growth of cells on plates that require expression of that specific reporter gene. When the two proteins interact, the promoter will be activated by the interaction of the proteins fused to the Gal4 DBD and AD. Growth indicates interaction between the two proteins, whereas lack of growth indicates no interaction.

Msi1p, when fused to the Gal4 DBD, has been found to activate transcription of the reporter gene, *HIS3* under the Gal4-activated promoter. This transcriptional activation of the promoter by Msi1p in the absence of the Gal4 AD has been termed “one-hybrid” activity because of the presence of only the single Gal4 DBD-Msi1p hybrid protein (SDJ, unpublished observations). One-hybrid activity of Msi1p is only present when *S. cerevisiae* with the Gal4 DBD-Msi1p is grown on nonfermentable carbon sources, such as glycerol. When grown on fermentable carbon sources (i.e. dextrose) Gal4 DBD-Msi1p does not activate transcription. All carbon sources that do not induce growth in the one-hybrid system of Msi1p are consumed fermentatively. Likewise, all inducing carbon sources must be consumed nonfermentatively (Table 1). This one-hybrid activation suggests that Msi1p

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<th>Inducing</th>
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<td>Glycerol</td>
<td>Dextrose</td>
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<td>Acetate</td>
<td>Fructose</td>
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<tr>
<td>Ethanol</td>
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<td>Galactose</td>
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Table 1- Known inducing and non-inducing carbon sources of the Msi1p one-hybrid activity.
acts as a transcription factor during aerobic growth conditions. Because dextrose is
catabolized anaerobically during normal growth conditions in *S. cerevisiae*, it is possible
Ms1p activation may be linked to a deviation from normal anaerobic growth.

During the diauxic shift, *S. cerevisiae* change from metabolizing dextrose
anaerobically and instead begin to aerobically metabolize ethanol, a byproduct of
fermentation. Microarray screens of *MSI1* mRNA during the diauxic shift shows no
change in expression (DeRisi et al., 1997). Thus, *MSI1* mRNA accumulation is likely
independent of carbon source stress. This leaves translational and post-translational
modification as likely modes of controlling Ms1p’s ability to accumulate during carbon
stress.

*S. cerevisiae* responds to environmental stresses (such as the diauxic shift from
anaerobic to aerobic growth, osmotic stress, heat and cold shock, and acidic and basic
conditions) in many ways, but most stress responses are activated by Msn2 and Msn4,
proteins that bind the DNA promoter stress response element (STRE). *MSI1* does not
have an STRE.

**S. cerevisiae stress response and Ms1p**

One hallmark of stress response is the accumulation of trehalose, a disaccharide
used to protect *S. cerevisiae* during environmental stress. In the Johnston laboratory, RT-
PCR analysis of the gene encoding one of the trehalose synthase protein, *TPS1*, in wild-
type *S. cerevisiae* grown in glycerol shows a strong induction of *TPS1* compared to the
same cells growing fermentatively in dextrose. *msi1Δ* *S. cerevisiae* cells grown in
glycerol show a failure to induce *TPS1* mRNA (Devin Miller, unpublished observation).
These results suggest Ms1p is necessary for transcriptional expression of *TPS1* mRNA in
glycerol, further suggesting Msi1p could be a carbon stress response protein. It is possible Msi1p may be a general stress response protein.

When *S. cerevisiae* is grown in conditions approaching freezing temperatures, trehalose synthesis increases, leading to a higher concentration of trehalose (Kandror *et al.*, 2004). This overall response to cold stress is very similar to heat shock, involving many of the same proteins, including the chaperone heat shock proteins. At 0°C to 4°C, the traditional heat shock proteins are induced in *S. cerevisiae* (Kandror *et al.*, 2004). Furthermore, *TPSI* and *TPS2* mRNA levels increase gradually between 10°C and 0°C peaking by 15-20 hours at 4°C (Kandror *et al.*, 2004). The increased levels of *TPSI* and *TPS2* mRNA result in greater accumulation of trehalose in less time than is observed during cell quiescence after the diauxic shift (Kandror *et al.*, 2004). With the speed at which heat shock adaptations take place, we have been unable to identify whether Msi1p increases in accumulation due to heat stress.

In this study, we are investigating the possibility that Msi1p accumulates as a response to cold stress. Kandror *et al.* has also shown *tps1Δ, tps2Δ* and *msn2Δ, msn4Δ S. cerevisiae* double mutant cells have decreased cell viability at 0°C that becomes very noticeable by 2 weeks when compared to wild-type cells (2004). This is due to the inability of *S. cerevisiae* to synthesize trehalose for protection against cold stress. If Msi1p is a stress response protein, or controls *TPSI* and *TPS2* mRNA expression we believe *msi1Δ S. cerevisiae* could have decreased cell viability at 0°C.

This article shows the one-hybrid activity of Msi1p is aided by the ability of the protein to accumulate in greater amounts in cells grown in non-fermentable carbon sources than in fermentable carbon sources. We provide evidence that the lower
accumulation of Msi1p is not the result of poly-ubiquitination of Msi1p suggesting post-transcriptional or translational control. We also provide evidence that suggests the newly identified novel function of Msi1p may be to act as a transcription factor of stress-induced genes. Furthermore, we believe this function could be linked to the ability of MSII to suppress the overactive RAS/cAMP pathway by reducing cAMP concentrations, resulting in an increase of HSPs and stress response. We hypothesize if Msi1p is involved in stress response, the MSII mRNA will exhibit similar accumulation as TPS1 and TPS2 mRNA during cold shock and heat shock (Kandror et al., 2004). If Msi1p is involved in more than one type of stress response, it is possible Msi1p could be a general stress response protein. We show further Msi1p accumulates in cold stress, but loss of msi1 does not lead to a loss of cell viability. Finally, Msi1p accumulation is independent of interactions with Msn2p and Msn4p.

Methods and Materials

Strains

S. cerevisiae strains used are all from the North Central College Yeast Laboratory Collection (y). Wild type (y1) is S. cerevisiae in a w303 background, MATa. Other strains are isogenic to y1, but y605 contains MSII::13 myc-HIS3MX6, and y859 contains msi1::hisG.

Culture and Lysis Conditions

Cultures were grown in either liquid yeast peptone dextrose (YPD) or yeast peptone glycerol (YPG) (Guthrie and Fink, 2002) overnight at 30°C with shaking. After growth, cultures were centrifuged to pellet cells at 2000 rpm for two minutes at 4°C. Cells were washed three times in sterile H2O. Cells were pelleted by centrifugation and
washed once in lysis buffer (10 mM Tris-Cl, pH 8.0, 150 mM NaCl, 10% glycerol, 0.05% Tween 20, 5 mM EDTA, with 1 mM DTT, 1 mM PMSF, 50 mM NaF, 0.2 mM Na$_3$VO$_4$). Cells were lysed in two packed cell volumes of lysis buffer and glass beads by vortexing at room temperature (25°C) for 5 minutes. Supernatants were collected from lysates centrifuged at 13,200 rpm for 2 minutes.

**Western Blotting**

Protein concentration in lysates was estimated by measuring absorbance at 280 nm. All lysates were normalized to 7.5 µl lysate for A$_{280}^{-}=0.120$. Lysates were run in an 8% SDS-PAGE at 150 V for one hour and transferred to nitrocellulose blot (Optitran BA-83) at 65V for one hour. Blot was blocked overnight in a 2% BSA solution in Tris-Buffered Saline Tween-20 (TBST) at 4°C.

Western blotting with the primary and secondary antibody occurred for one hour each with the antibody concentrations listed (Table 2). Dilutions were made in TBST. Bound antibodies were identified with SuperSignal West Dura Extended Duration Substrate (Pierce).

| Table 2- Antibodies and concentrations used for each western blot experiment. |
|-----------------|-----------------|-----------------|-----------------|
| **Experiment**  | **Primary Antibody** | **Secondary Antibody** | **Dilution of Antibodies** |
| Cold Stress     | Mouse anti-myc (Santa Cruz) | Goat anti-mouse HRP (Santa Cruz) | 1:40,000 |
| Msn2            | Goat anti-Msn2 (Santa Cruz) | Rabbit anti-goat HRP (Santa Cruz) | 1:20,000 |
| Msn4            | Goat anti-Msn4 (Santa Cruz) | Rabbit anti-goat HRP (Santa Cruz) | 1:20,000 |
| Proteasome Inhibitor | Mouse anti-myc (Santa Cruz) | Goat anti-mouse HRP (Santa Cruz) | 1:40,000 |
| Ubiquitin       | Rabbit anti-Ub (Calbiochem) | Goat anti-rabbit HRP (Santa Cruz) | 1:20,000 |
Accumulation Test

Cultures of y1 and y605 were grown overnight at 30°C in YPD, YPG, yeast peptone acetate (YPA), yeast peptone ethanol (YPE), or yeast peptone fructose (YPF) (Guthrie and Fink, 2002).

Stress and Inhibitor Treatments

To test the effect of cold stress on Msilp accumulation, y1 and y605 were grown overnight in either liquid YPD or YPG at 30°C with shaking. Cultures were transferred to 3°C with shaking for 1, 2, 4, 6, 8, or 10 hours.

To test cell viability at 3°C, y1 or y859 were grown only in YPD overnight. Cultures were transferred to 3°C and samples were removed from each culture at 1, 2, 3, and 4 weeks. Samples were serial diluted and plated on SDC plates (Guthrie and Fink) in duplicate and incubated at 30°C for 3 days.

We investigated accumulation of Msilp after proteasome inhibition by treating an overnight growth of y1 and y605 with 10 μM lactacystin dissolved in DMSO or an equal volume of control DMSO. Cells were incubated at 30°C for an additional one hour with shaking. Cultures were lysed as described above.

Attachment of ubiquitin to Msilp after lactacystin treatment was also investigated. Cultures of y1 or y605 were grown in YPD or YPG overnight at 30°C and then treated with lactacystin or DMSO as described. Cells were lysed as described before. Lysates were immunoprecipitated for Msilp by incubating at 4°C with 1:2,000 mouse anti-myc antibody (Santa Cruz) for two hours and with 1:400 Protein G agarose beads (Santa Cruz) for an additional two hours at 4°C with mixing. Beads were washed three times in lysis buffer with centrifugation and removal of supernatants between
washes. Msi1p was eluted from the beads by incubation with 20 μl of SDS Buffer containing 1 mM DTT at 65°C for 20 minutes.

Results

Msi1p accumulates in cells grown in nonfermentable carbon sources

Msi1p has a characteristic in which it can induce one-hybrid growth (acting as a transcription factor) when grown on nonfermentable carbon sources, but not on fermentable carbon sources. We hypothesize the one-hybrid activity of MSII is directly related to accumulation of Msi1p. To determine the cause of the one-hybrid carbon source dependence, S. cerevisiae was grown in fermentable and nonfermentable carbon sources and cell lysates were run on a SDS-PAGE. Western blotting revealed Msi1p levels increase about 2.5 to 3-fold in nonfermentable carbon sources above the level found in fermentable carbon sources (Figure 1a). Western blot analysis also shows there

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**Fig. 1a** - Accumulation of Msi1p in nonfermentable carbon sources at 30°C. Lanes contain equal amounts of total protein from: (1) Wild type S. cerevisiae (y1); (2) S. cerevisiae MSII::13 myc-HIS3MX6 (y605), YPD; (3) y605, YPF; (4) y605, YPG; (5) y605, YPA; (6) y605, YPE.

**Figure 1b** - Accumulation of Msi1p with addition of fresh media at 30°C. Lanes contain equal amounts of total protein from: (1) y1 YPD, (2) y605 YPD, (3) y605 YPD + fresh YPD 4 hr., (4) y605 YPG.
is no sign of post-translational modification. If there were modification to the protein, the band would likely show reduced mobility on the western blot (Figure 1a). Msil1p from cells grown in both fermentable and nonfermentable carbon sources are migrating the same distance in the SDS-PAGE, which shows Msil1p is the same mass in any carbon source condition (Figure 1a). The ability of Msil1p to induce one-hybrid activity in nonfermentable carbon sources may be caused by the increase in Msil1p accumulation.

With S. cerevisiae growing overnight in dextrose, we worried cells could be reaching the diauxic shift, leading to the increased accumulation of Msil1p as cells began to metabolize ethanol aerobically. Ethanol is a waste product of dextrose fermentation. To determine whether Msil1p accumulation in dextrose was from ethanol metabolism, fresh dextrose was added to cultures of S. cerevisiae grown overnight in dextrose, and cells were allowed to grow for 4 additional hours. Western blotting of Msil1p showed levels of Msil1p after fresh dextrose was added decreased slightly below that observed after overnight growth in dextrose (Figure 1b). We have not been able to completely remove the presence of Msil1p completely suggesting Msil1p is expressed at some basal level within S. cerevisiae regardless of carbon source.

We attempted to determine if the Msil1p accumulation in S. cerevisiae grown in dextrose was dependent upon some aerobic growth. We treated S. cerevisiae growing in dextrose overnight with cyanide to block the electron transport system, but saw no change in the Msil1p accumulation in dextrose (unpublished observations).
Ubiquitin attachment to Msi1p

The mechanism controlling Msi1p accumulation is not known. We do not believe the accumulation difference during carbon stress is due to transcriptional or post-transcriptional control because the DeRisi et al. microarray analysis shows MSII expression to be continuous during the diauxic shift (1997). With no post-translational modification of Msi1p detectable by western blot analysis, Msi1p could be controlled by increased degradation during fermentable growth. We hypothesized if Msi1p was being degraded by the 26S proteasome in dextrose culture, then treatment of S. cerevisiae with proteasome inhibitor should lead to an increase of Msi1p in dextrose over time. Western blot analysis of Msi1p shows there is no change in the Msi1p accumulation when treated with lactacystin in dextrose or glycerol (Figure 2a).

Lane 1 2 3 4 5 6

Figure 2a- Msi1p with proteasome inhibitor treatment.
Normalized protein concentrations of: (1) y605 YPD, (2) y605 YPG, (3) y605 YPD + DMSO, (4) y605 YPG + DMSO, (5) y605 YPD + lactacystin, (6) y605 YPG + lactacystin.
To confirm that Msi1p is not being ubiquitinated and degraded by the 26S proteasome, *S. cerevisiae* was treated with lactacystin, Msi1p was immunoprecipitated, and ubiquitin was detected by western blotting. If Msi1p were interacting with ubiquitin, we would have expected to see ubiquitin in the western blot present after immunoprecipitation of Msi1p. The results show that without immunoprecipitation and after lactacystin treatment, ubiquitin is present within the crude lysate (Lane 2, Figure 2b). After immunoprecipitation for Msi1p, there is no ubiquitin remaining in any lysate. Absence of ubiquitin from Msi1p immunoprecipitation suggests Msi1p is not tagged by ubiquitin for degradation by the 26S proteasome.

Together, Figures 2a and 2b provide insight on how Msi1p accumulation is controlled. Inhibition of the 26S proteasome does not lead to an increased accumulation
of Msilp in fermentable carbon sources (Figure 2a). Furthermore, Msilp is not polyubiquitinated and degraded by the 26S proteasome (Figure 2b).

**Cold Stress Response of Msilp**

Msilp accumulates during carbon stress (Figure 1a). We investigated whether Msilp also accumulates during cold stress. Western blot analysis of Msilp revealed a modest general increase in accumulation of Msilp in dextrose-containing media during increasing times of exposure to cold stress at 3°C (Figure 3a). We chose to expose cultures to the 3°C environment for 10 hours because Kandror et al. show that induction of TPS1 and TPS2 mRNA occurs at 15-20 hours at 4°C (2004). We believe if Msilp controls TPS1 expression then Msilp accumulation will occur during the first few hours before TPS1 mRNA expression. Over the 10-hour time period *S. cerevisiae* were exposed to the cold stress, Msilp accumulation gradually increased, peaking at 4 hours and remaining relatively constant for the remaining time (Figures 3a and 3c). Other western blots have shown Msilp to peak at 8 hours in dextrose, and then begin to decrease (data not shown). Msilp from *S. cerevisiae* grown in glycerol and then exposed to the cold stress shows an increase in accumulation, much like we see in dextrose (Figure 3b). In glycerol, Msilp levels increase for up to 6-8 hours, and then begin to decrease (Figure 3c). This suggests Msilp may be activated by growth in glycerol before accumulation in cold stress response, and then degraded by a possible feedback mechanism, or other mode of control. As is the case with the dextrose cold stress experiment, we cannot determine whether Msilp levels continue to decrease, or are controlled in a sinusoidal manner.
Cell Viability of *S. cerevisiae* lacking *msi1* at 3°C

Kandror *et al.* shows loss of both *tpsl*, and *tps2* in *S. cerevisiae* leads to a loss in cell viability over a 3 week period at 0°C (2004). Cell viability also decreases for loss of *msn2* and *msn4*, but the loss is not as dramatic as for *tpslΔ* and *tps2Δ* *S. cerevisiae* (Kandror *et al.*, 2004). Wild type *S. cerevisiae* viability was constant throughout the 21-day experiment (Kandror *et al.*, 2004). We set out to explore whether loss of *msi1* from *S. cerevisiae* would lead to a loss in cell viability. During the first two weeks, cell viability in both wild type and *msi1Δ* *S. cerevisiae* increased (Figure 4). We believe cultures did not reach saturation before transfer to 3°C, allowing cultures to continue to
grow to saturation. We allowed our experiment to continue for an additional 7 days to make up for this issue. Viability of \textit{msi\textDelta{} S. cerevisiae} is similar to that of wild type \textit{S. cerevisiae}, suggesting loss of \textit{msi1} does not lead to a loss in cell viability (Figure 4).

\textit{MSII} is not essential for cell survival during cold stress.

\textbf{Msn2 and Msn4 do not interact with Msi1p}

We hypothesized Msi1p could be a stress response protein because the protein accumulates during both carbon stress and cold stress. Msn2 and Msn4 are two important stress response proteins that activate transcription of genes with stress response elements (STREs). Western blotting of immunoprecipitated Msi1p shows Msn2 and Msn4 do not bind with Msi1p during carbon stress (Figure 5a and 5b).

There was a possibility Msi1p was not immunoprecipitated during the process. To test this, we western blotted immunoprecipitate lysates for presence of Msi1p. Western
Figure 5a- Immunoprecipitation of Msi1p while and western blotting for Msn2. (1) y605 YPD crude lysate, (2) y1 YPD no antibody IP, (3) y1 YPD IP, (4) y605 YPD no antibody IP, (5) y605 YPD IP, (6) y605 YPG no antibody IP, (7) y605 YPG IP.

Figure 5b- Immunoprecipitation of Msi1p while and western blotting for Msn4. (1) y605 YPD crude lysate, (2) y1 YPD no antibody IP, (3) y1 YPD IP, (4) y605 YPD no antibody IP, (5) y605 YPD IP, (6) y605 YPG no antibody IP, (7) y605 YPG IP.

Figure 5c- Immunoprecipitation of Msi1p while western blotting for Msi1p. (1) y1 YPD no antibody, (2) y605 YPD no antibody, (3) y605 YPD no antibody, (4) y605 YPD IP, (5) y605 YPD IP, (6) y605 YPG no antibody IP, (7) y605 YPG IP, (8) y605 YPG IP.
blot analysis shows Msilp was effectively immunoprecipitated from y605 cultures in both dextrose and glycerol containing media (Figure 5c). Msilp was not immunoprecipitated in the y1 control, or in the absence of the anti-myc antibody. These results show Msn2 and Msn4 do not interact with Msilp when cells are grown in dextrose or glycerol.

Discussion

Msilp accumulates in cells grown in nonfermentable carbon sources

The one-hybrid activity of Msilp is likely caused by the increased accumulation of Msilp in nonfermentable carbon sources (Figure 1a). It is not understood why there is no activation seen in fermentable carbon sources even though Msilp is present at some level. Addition of fresh dextrose did not dramatically change the accumulation of Msilp (Figure 1b). This suggests accumulation of Msilp in dextrose is not completely due to diauxic shift in dextrose after overnight growth. Rather, Msilp might be accumulating at some basal level within *S. cerevisiae* at all times. It may be useful to have others look at this minimal accumulation of Msilp, especially in after cyanide treatment.

The question that immediately rises is what concentration of Msilp is needed in order for the one-hybrid activity to occur? Furthermore, is there another protein that must interact with Msilp in nonfermentable conditions that cause the one-hybrid activity to occur? The ability of Msilp to accumulate in multiple nonfermentable carbon sources suggests this trend is true for all nonfermentable carbon sources and accounts, at least in part, for the one-hybrid activity that has been previously noted. These two results suggest the carbon stress induced by aerobic growth activates Msilp’s ability to act as a transcription factor by increasing the overall accumulation of Msilp in *S. cerevisiae* cells.
Models of Msi1p transcriptional activity

We hypothesize the ability of MSI1 to induce one-hybrid growth on non-fermentable carbon sources is directly related to the accumulation of Msi1p. At this time, there are three possible models by which Msi1p could be acting as a transcription factor. Transcriptional activity induced by Msi1p, such as the one-hybrid activity, could be based on concentration of Msi1p within *S. cerevisiae*. The ability of Msi1p to reach a certain threshold concentration (as is the case in nonfermentable carbon sources) would be great enough to cause the observed one-hybrid activity. If this is the case, Msi1p may have weak binding to some other factor, and thus a greater concentration or accumulation of Msi1p is needed in order for binding to the factor to occur in a constructive manner resulting in transcriptional activation, as seen on nonfermentable carbon sources. Higher concentrations of Msi1p would increase the time, or rather the frequency with which Msi1p could be in contact with proteins needed to bind.

A second possible model would involve the mechanism of the previous example, but Msi1p would compete for binding to the regulatory factor with an inhibitory protein. When this inhibitor protein binds to the regulatory factor it would inhibit Msi1p transcriptional activity. At high concentrations, Msi1p could out-compete the inhibitor, bind effectively to the regulatory factor, and activate transcription.

In mammalian cells, hemoglobin is a quaternary protein that homotetramerizes. Hemoglobin binds oxygen and supplies the oxygen to other cells within the mammalian body. Binding of oxygen to hemoglobin is a cooperative process, in which binding of one oxygen molecule to a heme group facilitates the binding of oxygen to another heme group (Horton *et al.*, 2002). Maybe Msi1p binds as a homodimer, or homo-oligomer
during carbon stress that facilitates the ability of Msi1p to activate transcription. This might explain the suddenness of the transition between minimal Msi1p accumulation in fermentable carbon sources and increased accumulation in nonfermentable carbon sources (Figure 1a). It is possible such a homo-oligomer form could stabilize Msi1p from destruction.

Finally, a third possibility is that Msi1p may be interacting with some factor during its one-hybrid activity that has yet to be detected. Msi1p may need to bind with another protein, or an activating factor for activation of transcription that is present during carbon stress. If this were the lone mode of regulation, a higher accumulation of Msi1p in nonfermentable carbon sources would not be needed for activation. This model would not be able to explain Figure 1a. What this model does explain is how Msi1p can bind to DNA to activate transcription. Without the Gal4 DBD, Msi1p cannot activate one-hybrid activity, showing the need for a factor that can identify DNA elements for Msi1p to induce transcription (SDJ, unpublished observations). Thus, Msi1p may bind to a protein or factor that can recognize the promoter sequence or Msi1-induced genes. Msi1p would act as the activation domain.

Regulation of Msi1p Accumulation

Western blot analysis of Msi1p with lactacystin treatment shows Msi1p does not accumulate more when the 26S proteasome is inhibited (Figure 2a). Furthermore, we have been unable to detect a poly-ubiquitinated Msi1p (Figure 2b). These results suggest the accumulation difference of Msi1p in fermentable and nonfermentable carbon sources is controlled by some process other than degradation on the proteasome. It is possible Msi1p accumulation is being controlled by an upstream regulator through transcriptional
regulation, but unlikely because of observations from DeRisi et al. that show MSII mRNA accumulation does not change during growth (1997). An upstream regulator of MSII that controls accumulation has yet to be found.

With these results and those from DeRisi et al., we must look to another mode of degradation. One possible mechanism is degradation of Msi1p by the lysosome. Another possible method of Msi1p control could be translational control. In this method, Msi1p could be controlled by a factor that is necessary for efficient translation of Msi1p. It is unlikely the eukaryotic initiation factor 2 (eIF2) in S. cerevisiae is controlling MSII translation because eIF2 is turned off during stress (Proud, 2004). This mode of regulation is contrary to Msi1p accumulation during carbon stress and cold stress. Furthermore, Msi1p stability could be controlled by the ability of Msi1p to bind to another protein or factor during stress, allowing greater accumulation of Msi1p. To determine such an interaction, we would have to either find a likely candidate and immunoprecipitate Msi1p with the hopes of identifying the candidate protein. The other option would be to run a non-denaturing PAGE, in order to identify a change in protein mass during either stress or no stress condition, suggesting binding of Msi1p to another protein or factor.

It's possible Msi1p is being stabilized by its location within the cell. Within mammalian cells, Hypoxia inducible factor-1 (HIF-1) is controlled in part by its location within the cell. During low oxygen, or hypoxic conditions, HIF-1 is found within the nucleus as a transcription factor. During normoxia, HIF-1 relocalizes to the cytoplasm, where it is degraded by the 26S proteasome. Msi1p could be controlled in a similar manner, where during stress conditions it is located within the nucleus and is protected
from degradation. During normal conditions, Msi1p could localize to the cytoplasm and become degraded by lysosomes. To determine localization of Msi1p during stress and no stress conditions, we would have to separate *S. cerevisiae* nuclei from cytoplasm and determine whether there is a difference in Msi1p accumulation in the two conditions.

We have not yet explored whether the half-life of Msi1p during growth in dextrose and glycerol differ. The control of half-life may explain the difference in accumulation observed during carbon stress. If this is the case, we must ask what modification is Msi1p undergoing to enhance or repress the half-life of the protein? We may want to also look at *MSII* mRNA levels during carbon stress in order to confirm or refute results of *MSII* mRNA expression shown by De Risi et al. (1997).

**Cold Stress Response of Msi1p**

Msi1p accumulates during cold stress in cells grown in either dextrose or glycerol. This tells us the cold stress response of Msi1p is largely independent of carbon source. Accumulation seems to take longer in dextrose than glycerol (Figure 3c). This may be due to higher accumulation of Msi1p in *S. cerevisiae* after growth at 30°C. If accumulation of Msi1p in glycerol is greater during physiological temperatures, then less Msi1p must accumulate in order for the protein to reach levels needed for its cold stress response. We hypothesize Msi1p levels begin to decrease in glycerol after 8 hours at 3°C. With the initial increase in accumulation of Msi1p at 0 hours at 3°C, Msi1p is able to activate cold stress response faster. As a result, the accumulation of Msi1p occurs for a shorter amount of time in glycerol than in dextrose (Figure 3b). Msi1p increases in accumulation at 3°C in dextrose above levels seen when grown at 30°C. The accumulation process of Msi1p in dextrose lasts longer at 3°C than in glycerol (about 2
hours longer), which may be due to a smaller quantity of Msi1p accumulated in dextrose before the cold stress. The accumulation of Msi1p in both dextrose and glycerol above levels seen at 30°C growth suggest Msi1p is responding to cold stress in S. cerevisiae.

In glycerol, Msi1p accumulation at 3°C begins to decrease at around 8 hours (Figure 3b). In dextrose, Msi1p accumulation at 3°C increases up to the 10-hour time point we have tested (Figure 3a). Because no experiment has been run for longer than 10 hours, we do not yet know what is happening beyond the 10-hour time point in the 3°C environment. We hypothesize results past 10 hours in dextrose may look like that of glycerol, where Msi1p accumulation will begin to decrease over time.

As of yet, we do not know what happens to Msi1p in S. cerevisiae during the cold stress after accumulation stops. We have only seen levels begin to decrease in glycerol (Figure 3b), but how levels decrease is not known. One question that must be answered is whether Msi1p accumulation acts differently in dextrose and glycerol after the initial accumulation (up to 10 hours) stops. One possible model may be that Msi1p levels gradually decrease as the protein (acting as a transcription factor) has possibly activated TPS1 and TPS2 transcription. After activation of these genes, Msi1p levels may decrease due to a feedback mechanism (possibly involving trehalose), or MSII is repressed, inhibiting production of new Msi1p. As trehalose increases, repression of MSII would continue until trehalose decreases in S. cerevisiae. Another model we propose is similar to the one above, but rather than completely repressing MSII the gene is instead controlled as a response to TPS1 and TPS2 mRNA in the cell. As Tps1p and Tps2p are degraded because of “old-age,” the MSII gene could be reactivated, allowing production of Msi1p. The half-life of Tps1p and Tps2p are not known, though. If this continues,
Msi1p levels would be controlled in a sinusoidal manner, or controlled in waves of accumulation and decay.

The question now becomes how is \textit{MSII} controlled? Recent results from the yeast laboratory suggest Msi1p is not ubiquitinated or degraded by the 26S proteasome (Figure 2). De Risi \textit{et al.} have shown \textit{MSII} expression before and after \textit{S. cerevisiae} diauxic shift remains constant. This suggests \textit{MSII} accumulation is independent of carbon stress. Whether \textit{MSII} expression is independent of cold stress has yet to be determined. It is possible \textit{MSII} is controlled during translation, but we have not been able to determine whether this is how accumulation of Msi1p is controlled during cold stress and carbon stress.

\textit{MSII} is not essential for cell viability during cold stress

We have shown \textit{msi1}Δ \textit{S. cerevisiae} survive during cold stress at the same rate as wild type \textit{S. cerevisiae} (Figure 4). This suggests \textit{MSII} is not essential for cell survival during cold stress. Results from Johnston \textit{et al.} show loss of \textit{msi1} in \textit{S. cerevisiae} during heat shock does not have any effect upon cell viability (2001). Because Kandror \textit{et al.} suggest cold stress and heat shock induce many similar pathways and gene expression, we believe our results can be considered consistent with those found in Johnston \textit{et al.} (2001). These results suggest \textit{MSII} may be behaving in a similar manner during both cold stress and heat shock. In that manner, Msi1p may be acting as a repressor of \textit{RAS} (Ruggieri \textit{et al.}, 1989).

\textbf{Msi1p does not interact with the stress response proteins Msn2 and Msn4}

We immunoprecipitated Msi1p and western blotted for Msn2 and Msn4 because we hypothesized Msi1p could be interacting with either stress response protein during
carbon stress. If Msn2 or Msn4 were found to interact with Msi1p, we would have been able to label Msi1p as a stress response protein and suggest a role as a coactivator of the stress response. Msn2 and Msn4 were not found to interact with Msi1p during growth in dextrose or glycerol (Figure 5). Because neither Msn2 or Msn4 seem to interact with Msi1p, we are uncertain whether Msi1p accumulation during carbon stress and cold stress are due to a general stress response, or through other signaling pathways. We could immunoprecipitate Msi1p and western blot for other stress response proteins to determine whether Msi1p interacts with any stress response proteins.

Msi1p does not have a STRE, suggesting it is not a protein that is transcriptionally regulated by Msn2 or Msn4. Rather, Msi1p could be an upstream regulator of Msn2 and Msn4 in carbon stress. These results further suggest the possibility that carbon stress and cold stress accumulation of Msi1p could be related to its control of RAS/cAMP pathway. By controlling this pathway, Msi1p can increase HSPs, allowing for adequate protection during stress. Furthermore, loss of msil leads to a loss of TPS1 mRNA levels during carbon stress (Devin Miller, unpublished observations). Loss of HSPs from msilΔS. cerevisiae may lead to loss of TPS1 mRNA. By deleting msil, RAS/cAMP is without this source of repression. This allows cellular growth while inhibiting HSP production (Ruggieri et al., 1989). We hypothesize the active pathway accounts for the lack of TPS1 expression, and adequate tolerance to stress. We must investigate the ability of Msi1p to interact with Msn2 and Msn4 during cold stress.

**Linking Msi1p accumulation to repression of RAS/cAMP pathway**

In S. cerevisiae, the RAS/cAMP pathway controls the response to growth. As the pathway is activated, cAMP activates protein kinase A (PKA), which in return
deactivates glycogen synthetase, promoting breakdown of glycogen for energy (Ruggieri et al., 1989). This results in a decrease of HSPs, leading to a loss of efficient cellular response to stress (Ruggieri et al., 1989). MSII was identified as a gene that when overexpressed could repress the RAS/cAMP pathway. Cells with an overactive RAS/cAMP pathway that were sensitive to heat shock became heat tolerant when MSII was overexpressed (Ruggieri et al., 1989). Repression of the RAS/cAMP pathway leads to an increased concentration of HSPs, allowing for heat tolerance. Finally, loss of msil did not have any effect on cell viability (Ruggieri et al., 1989).

Kandror et al. states many of the genes expressed during heat stress are also expressed during cold stress (2004). We have found Msi1p accumulation to increase during cold stress for at least the first 10 hours in dextrose and the first six in glycerol. It is possible the increase in Msi1p is repressing the RAS/cAMP pathway as it does during heat stress. This allows for increased concentrations of HSPs, and cell tolerance to cold stress. Furthermore, loss of msi1 during cold stress does not affect cell viability, suggesting very similar results to those found by Ruggieri et al. during heat shock (1989).

These results suggest Msi1p during cold stress is activated in order to repress the RAS/cAMP pathway, allowing for adequate protection to the stress. Whether this is the same effect that happens during carbon stress is not yet known. This can actually be resolved by testing one-hybrid activity of Msi1p during cold stress and heat stress to compare those results with carbon stress in order to determine whether either temperature stress induces one-hybrid activity.
Proposed model for Msi1p activity

Accumulation of Msi1p during carbon stress and cold stress may be controlled under a similar pathway. It is as probable, if not more likely, that Msi1p accumulation during carbon stress and cold stress are two separate pathways that are regulated by a determining factor. This factor could act as a switch between the activity of Msi1p during carbon stress and cold stress depending upon the correct environmental cue (Figure 6). If the stress is caused by carbon source, Msi1p will be activated. This activation will result in transcription of genes, possibly including TPS1. This may require another protein to bind DNA. It is also possible Msi1p homodimerizes. If cold stress is sensed, Msi1p will be activated by a separate pathway and will repress RAS. This will allow for activation of HSPs to protect against cold stress (Figure 6).

We hypothesize this is the likely scenario because Kandror et al. show many of the heat shock proteins also are activated during cold stress (2004). Thus, it is likely cold stress and heat shock responses are controlled by the same pathway. MSII is needed for suppression of the RAS/cAMP pathway, which leads to an increase of HSPs, and protection against heat shock sensitivity. Furthermore, MSII is not essential for cell viability during heat shock or cold stress (Figure 4).

Future Aims

From these findings emerge many questions regarding how Msi1p is regulated and how the protein interacts during different stresses in S. cerevisiae. One immediate question that must be answered is whether activation of Msi1p by carbon stress and cold stress act through similar or diverging signaling pathways. We believe this can be answered through a one-hybrid assay of Msi1p combining both carbon stress and
temperature stress at 3°C (cold stress), 30°C, and 37°C (heat shock). Heat stress does not induce one-hybrid growth on dextrose (SDJ, unpublished observations). We hypothesize that if cold stress does not induce one-hybrid growth on dextrose, cold stress activation of Msi1p may be acting in a similar pathway to Msi1p activity during heat shock. If cold stress does induce one-hybrid activity then cold stress may be signaling Msi1p in a manner similar to carbon stress. Preliminary results have shown the *S. cerevisiae* strain we are using for one-hybrid assays is unable to grow at 3°C. Thus, we must determine an experiment that will allow us to grow the *S. cerevisiae* without influencing the results of the experiment.

We must be able to determine whether Msi1p is accumulating during heat shock. Because activation of heat shock genes occurs quickly, we have been unable to determine whether Msi1p accumulates during heat shock in dextrose or glycerol. We must identify a time period that Msi1p is accumulating during heat stress (if any such time period exists). If we can show Msi1p is activated during heat stress, we may be able to link the converging evidence of cold stress and heat shock activity of Msi1p under one signaling pathway.

How is Msi1p being regulated? We have yet to determine a mechanism by which Msi1p is being regulated. One option is to test the expression of *MSI1* mRNA to see if *MSI1* is controlled by transcription regulation. We may want to invest time in looking at the half-life of Msi1p in dextrose and glycerol to determine whether half-life changes under carbon stress. To do this, we may look into collaborating with another laboratory that has the ability to use radioactive amino acids in order to detect Msi1p stabilization over time. We could use drugs to shut down translation in *S. cerevisiae* and then look at
the decay of Msi1p over time in dextrose in glycerol. This process is not as clean as using radioisotopes because treating *S. cerevisiae* with drugs will shut down translation of all proteins, which will eventually kill the cells.

What gene or genes (if any) regulate *MSII* transcription? We have not been able to detect genes that lead to the loss of the Msi1p transcriptional activity in *S. cerevisiae*. We may want to look at regulators of the *S. cerevisiae* stress response in order to determine whether *MSII* is involved within any of the stress response pathways of *S. cerevisiae*.

Finally, does Msi1p interact with other proteins? Msi1p cannot induce transcription alone (SDJ, unpublished observations), which leads us to hypothesize Msi1p must interact with another protein to bind DNA and activate transcription during carbon stress. We now know Msi1p does not interact with Msn2 or Msn4 during carbon stress. Does Msi1p interact with either Msn2 or Msn4 during cold stress? It will be to our advantage to immunoprecipitate Msi1p during cold stress to determine whether Msn2 or Msn4 are pulled down with Msi1p. Does Msi1p interact with or does *MSII* expression require *BCY1*, or *TPK1/2/3* for stress response and/or trehalose accumulation? *BCY1* and *TPK1/2/3* form a complex of proteins, PKA, that are required for activation of trehalose accumulation, entry into stationary phase, stress responses, and inhibition of cellular growth (Estruch, 2000).

The future of studying *MSII* looks to be promising because we have found Msi1p can activate transcription during carbon stress in *S. cerevisiae*, as well as accumulate during both carbon and cold stress. In future work, we plan to focus upon the differences and similarities between the carbon and cold stress accumulations of Msi1p. In this
process, the focus must be to determine proteins that interact with Msi1p or proteins that regulate \textit{MSI1} expression during these stress conditions.

Figure 6: Msi1p accumulation during carbon stress and cold stress could act under one similar pathway or separate pathways. Here, Msi1p is controlled by an environmental stress, activating a response factor and determinatin factor that determines whether Msi1p is to act as a transcription factor (carbon stress) or as a repressor of the \textit{S. cerevisiae} RAS/cAMP pathway.
Works Cited


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