A CHEMICAL-GENOMICS STUDY IDENTIFIES SNF1 AS A REPRESSOR OF GCN4 TRANSLATION

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Running Title: Snf1 regulation of Gcn4

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The Saccharomyces cerevisiae Snf1 kinase plays a critical role in recalibrating cellular metabolism in response to glucose depletion. Hundreds of genes show changes in expression levels when the SNF1 gene is deleted. However, cells can adapt to the absence of a specific gene when grown in long-term culture. Here we apply a chemical genetic method to rapidly and selectively inactivate a modified Snf1 kinase using a pyrazolopyrimidine inhibitor. By allowing cells to adjust to a change in carbon source prior to inhibition of the Snf1 kinase activity, we identified a set of genes whose expression increased when Snf1 was inhibited. Prominent in this set are genes that are activated by Gcn4, a transcriptional activator of amino acid biosynthetic genes. Deletion of Snf1 increased Gcn4 protein levels without affecting its mRNA levels. The increased Gcn4 protein levels required the Gcn2 kinase and Gcn20, regulators of GCN4 translation. These data indicate that Snf1 functions upstream of Gcn20 to regulate control of GCN4 translation in S. cerevisiae.

Snf1 is the catalytic subunit of a trimeric kinase complex and is one of the founding members of the AMP-activated protein kinase (AMPK) family that is present in all eukaryotic cells. AMPK serves as a nutrient sensor that is activated during times of nutrient and energy limitation (1). Once activated, Snf1/AMPK phosphorylates enzymes and transcription factors that recalibrate metabolism to conserve energy. In yeast, Snf1 is most active when cells are grown under glucose-limiting conditions where it promotes gluconeogenesis and utilization of alternative carbon sources (2). Earlier studies that compared transcript profiles of cells expressing Snf1 with those lacking Snf1 identified a large number of genes whose expression is Snf1-dependent (3). As expected, many of the Snf1-regulated genes participate in carbohydrate transport and metabolism. Furthermore, the Snf1-mediated regulation of transcription factors and other regulatory proteins may explain its global effect on gene expression (3). In this study, we created an analog-sensitive allele of the S. cerevisiae SNF1 gene that could be rapidly inactivated upon exposure to a pyrazolopyrimidine inhibitor (4). In contrast to using a snf1Δ mutation, chemical inhibition of Snf1 allowed us to identify genes whose expression is controlled by the kinase activity of Snf1 and not some other function of the protein. Using this system and genome-wide mRNA analysis, we identified a large number of genes whose expression is negatively regulated by Snf1. A common feature of many of these Snf1-repressed genes is their dependence on the Gcn4 transcriptional activator for proper expression.

In response to amino acid limitation, Gcn4 activates the transcription of a large number of genes that encode proteins involved in amino acid biosynthesis (5). The regulation of Gcn4 itself lies primarily at the level of GCN4 mRNA translation (6, 7). During amino acid starvation, uncharged tRNA...
molecules bind and activate the Gcn2 kinase, which phosphorylates the translation initiation factor eIF2α. Phosphorylation of eIF2α promotes GCN4 translation by inhibiting translation of four small open reading frames (uORFs) in the 5' regulatory region of the GCN4 message. Once translated, the stability of the Gcn4 protein provides a second level of control (7). In this study, we identified a role for Snf1 in repressing the translation of GCN4 mRNA, revealing a previously unrecognized mode of regulation for GCN4 translation and further illuminating the crosstalk between regulatory pathways that respond to amino acid and carbon source availability.

**EXPERIMENTAL PROCEDURES**

**Media and growth assays** – Synthetic complete media (SC) were prepared as described (8); synthetic complete medium lacking uracil (SC-Ura) was used to maintain plasmid selection as indicated. Halo assays were performed as described (9). For growth curves, cultures were grown overnight to saturation in SC-Ura medium containing 2% glucose and then diluted in SC-Ura medium containing 2% raffinose and 0.05% glucose to an OD600 of 0.1. Cultures were grown until the OD600 reached 0.3, divided and incubated with either dimethylsulfoxide (Me2SO) or 25 µM 2NM-PP1 dissolved in Me2SO, and OD600 values were recorded every 30 min.

**Yeast strains** – The *S. cerevisiae* strains used in these experiments are listed in Table 1. All strains have the same genetic background as FY2, a GAL2* derivative of S288C (10). FY1193 was obtained from Fred Winston. MSY strains have been previously described (11). KY and PY strains were constructed by genetic mating and tetrad dissection or integrative transformations. To generate strains containing the gcna::KANMX4 mutation or the gcna1::KANMX4 mutation, oligonucleotides were designed to amplify by PCR the alleles from the appropriate strain in the *S. cerevisiae* deletion collection (12). The purified PCR products were transformed into diploid strains heterozygous for snf1A and processed for tetrad analysis. The GCN4-HA::KANMX6 and gcna::KANMX4 strains were also constructed by PCR amplification of the appropriate fragments (13, 14) and transformation into diploid strains heterozygous for snf1A. Strains containing multiple kanamycin resistance genes were confirmed by genetic and PCR analyses.

**Plasmids** – The analog-sensitive allele of SNF1, snf1-as, contained in plasmid pSNF1-1132G-316, was made by Quick-Change mutagenesis (Stratagene) of pSNF1-316 (15). The plasmid encoding Gcn4c-13myc, pPS127, was generated by gap repair (16) from p238 (17). p238 is derived from p164 (18), which is a YCp50-based (CEN/ARS, URA3) plasmid expressing Gcn4, but p238 also contains the point mutations in the upstream open reading frames (uORFs) described by Mueller et al. (19). The p238 plasmid was digested with *Afl*III and *Xba*I and the 9.6 kb fragment was transformed into PY1017, a strain that contained a GCN4-13myc::KANMX6 allele, previously created by PCR-mediated one-step gene replacement (13). In some experiments, we needed to use a 13myc epitope, instead of the HA tag, because expression of Gcn4c (untagged or tagged) in strains lacking a chromosomal copy of GCN4 induced expression of an unknown protein that was recognized by HA antibody in western blot analysis and migrated at a molecular weight similar to Gcn4 in SDS-PAGE gels. Gap-repaired pPS127 plasmids (16) were isolated and confirmed by sequencing the GCN4 uORFs and the 3' junction with the 13myc epitope tag. The plasmid encoding Gcn4-13myc, pPS128, was created by substituting the *Afl*III-*Xba*I fragment from pPS127, encoding the C-terminus of Gcn4 and 13myc tag, into the same sites in p164 (18). GCN4-lacZ plasmids p180 and p227 have been previously described (18, 19). Oligonucleotide and plasmid sequences are available upon request.

**Yeast microarray analysis** – Yeast cultures were grown in triplicate to an OD600 of 0.8 to 1.0 at 30 °C in SC-Ura medium containing 2% glucose. Cultures were shifted to medium containing 2% raffinose and 0.05% glucose for two hours. The drug 2NM-PP1 was added to cultures to a final concentration of 25 µM for 40 min prior to harvesting. Total RNA samples were prepared from cells by glass bead lysis, digested with DNase and purified (RNeasy Kit-Qiagen). Sample quality was assessed by agarose gel electrophoresis using a BioAnalyzer (Agilent). Triplicate RNA samples were combined and 7 µg of the yeast RNA was used to synthesize cDNA using the SuperScript Double-Stranded cDNA Synthesis Kit (Invitrogen). Biotin-
labeled RNA probes were prepared using the BioArray High Yield RNA Transcript Labeling Kit (Enzo). The biotin-labeled RNA probe (15 µg) was fragmented and hybridized to an Affymetrix Yeast Genome S98 Array. Arrays were washed using an Affymetrix GeneChip Fluidics Station 450. DAT files were generated using Microarray Analysis Suite 5.0 (MAS5). Values from duplicate arrays were compared and found to be highly reproducible. Spot fluorescence intensity values ranged from 0 to 5.7 with a standard deviation of 0.38 and 0.43 for the duplicate sets. The Affymetrix data discussed in this manuscript have been deposited in NCBI’s Gene Expression Omnibus (GSE12061) and are accessible through GEO Series accession number GSE12061 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE12061). In addition, normalized values are given in Supplementary Table 1.

Northern blot analysis – Cells grown at 30 °C as described in the figure legends were harvested by centrifugation, and the pellets were frozen immediately by immersion in liquid nitrogen. Cell pellets were thawed in the presence of RNA lysis buffer and processed as described (21). Hybridization probes were prepared by PCR and random-prime labeling (16).

Western blot analysis – Cells grown at 30 °C as described in the figure legends were harvested as described for the northern blot analysis. Cell pellets were thawed in the presence of lysis buffer (22), containing complete protease inhibitor (Roche) and 10 mM NaF, and processed as described (23), except that 50 µg of total extract was separated on an SDS-10% polyacrylamide gel. The HA epitope antibody (Roche) was used at a 1:3000 dilution, and the c-myc epitope antibody (Covance) was used at a 1:500 dilution. Antibody directed against Sse1, a component of the Hsp90 chaperone complex, was used at a 1:2000 dilution as a loading control (gift from Jeff Brodsky, University of Pittsburgh). Western blot experiments were performed two to five times with extracts prepared from independently grown cultures. Representative results are shown.

β-galactosidase assays - Extract preparations, β-galactosidase assays, and unit calculations were performed as described (24). Values represent the average of two to four transformants for each plasmid assayed at two different extract concentrations. Standard errors ranged from 3 to 22%.

RESULTS

Development of an analog-sensitive allele of SNF1 – To study the immediate downstream targets of the Snf1 kinase, we sought a means to rapidly and selectively inhibit the activity of Snf1 in vivo. To this end, we chose to employ a chemical genetic method that enabled rapid, potent and selective inhibition of the kinase activity of Snf1 by an enlarged analog of the pyrazolopyrimidine-based kinase inhibitor PP1, through introducing a space-creating mutation at the conserved gatekeeper position within the kinase domain (4). The gatekeeper residue I132 in the Snf1 kinase domain was changed to glycine, creating the snf1-as allele. The Snf1-I132G protein is fully functional as judged by its ability to complement a deletion of the SNF1 gene for growth on alternative carbon sources, growth in the absence of inositol, and the induction of invertase (data not shown). To identify a PP1 analog that could specifically inhibit Snf1 kinase activity, cells expressing either wild-type Snf1 or Snf1-I132G were tested for the ability to grow on medium containing sucrose in the presence of a panel of PP1 analogs (Fig. IA and data not shown). Among the 22 compounds tested, the molecule 2NM-PP1 most effectively inhibited growth of snf1-as cells without affecting growth of SNF1 strains. The functionality of the Snf1-I132G variant and the selectivity of inhibition by 2NM-PP1 were assessed by a more sensitive assay in which the growth rate of cells was monitored in raffinose medium (Fig. 1B). Snf1 kinase function is required for cells to utilize raffinose as a carbon source. The growth rate of the snf1-as (Snf1-I132G) strain but not the wild-type strain was reduced in the presence of 2NM-PP1, indicating that 2NM-PP1 is specific for the Snf1 variant. Furthermore, in the absence of 2NM-PP1, the snf1-as strain showed a growth rate comparable to the wild-type strain, indicating that the I132G substitution does not itself affect Snf1 function.

To further characterize the Snf1-I132G variant, we examined the Snf1-dependent induction of SUC2 mRNA by northern blot analysis (Fig. 2). In cells expressing wild-type Snf1, we detected a large increase in the abundance of SUC2 mRNA...
relative to ACTI mRNA upon shifting cells from glucose to raffinose (lane 2). The addition of the inhibitor 2NM-PP1 did not affect the induction of SUC2 mRNA in wild-type cells (lanes 7 and 8). In the absence of 2NM-PP1, induction of SUC2 mRNA in the Snf1-1132G strain (lane 10) was indistinguishable from induction in the wild-type strain (lane 2). However, addition of 2NM-PP1 to raffinose-grown Snf1-1132G cells returned SUC2 mRNA levels back to the uninduced state (lanes 15 to 22). A titration of the inhibitor showed that 25 µM 2NM-PP1 was sufficient to abrogate Snf1-1132G function. Lower concentrations of inhibitor or shorter incubation times only partially reduced SUC2 transcription (data not shown). Based on the results of the growth assays and SUC2 northern blot analyses, incubation with 25 µM 2NM-PP1 for 40 min was used to inhibit the activity of Snf1-1132G in subsequent experiments. Taken together, our data show that the combination of the Snf1-1132G variant and the pyrazolopyrimidine compound 2NM-PP1 provides us with the ability to efficiently and selectively inhibit the Snf1 kinase in vivo.

Identification of Snf1 regulated mRNAs

The Snf1-1132G variant allowed us to first grow cells in conditions where Snf1 was activated and then selectively inhibit its kinase activity by the addition of 2NM-PP1. We reasoned that this approach would enhance our ability to identify Snf1-repressed genes, a class of genes that had not been well studied. Snf1 activity was first stimulated by growing cells for two hours in medium containing raffinose and then inhibited by addition of 2NM-PP1. RNA was harvested 40 min following addition of the inhibitor. At this time, the growth rate of these cells had not yet changed (Fig. 1B), although SUC2 transcription was greatly reduced (Fig. 2). Therefore, any effects on mRNA levels should not be due to the cells undergoing growth arrest. Using Affymetrix yeast genome microarrays to detect mRNA transcript levels, 153 genes showed a greater than 2.8-fold increase in mRNA abundance in the snf1-1As strain treated with 2NM-PP1 compared with the SNF1 strain treated with 2NM-PP1 (Fig. 3A and Supplementary Table 1). These genes were categorized by their gene ontology (GO) annotation to determine if any biological processes were enriched in this set of genes (25). Remarkably, 35% (P-value of < 1 x 10^-2) of the genes repressed by Snf1 clustered to the GO-ID 6519, "amino acid and derivative metabolic process."

Gcn4-regulated genes are repressed by Snf1. – Since Gcn4 is known to activate the transcription of many amino acid biosynthetic genes, we asked if any of the 153 genes up-regulated upon acute inhibition of Snf1 kinase activity are targets of Gcn4 (5). We compared our data with two previously published sets of Gcn4-regulated genes. The first set contains those genes whose transcription decreased 1.5-fold or more when GCN4 was deleted ((26) and data downloaded from http://transcriptome.ens.fr). Importantly, similar to our conditions, these data were obtained from cells grown in synthetic complete media as opposed to media with limited amino acids, which has been used in other studies (5). Of the 117 genes whose proper expression requires Gcn4 activity (26), 54 were up-regulated in the inhibitor-treated snf1-as strain (Fig. 3B). The second set we examined was compiled by Pokholok et al. (27), who generated a list of 84 Gcn4 target genes using the following criteria: 1) their expression changed in a Gcn4-dependent manner during amino acid starvation; 2) their promoters contained a conserved Gcn4-binding site; and 3) their promoters were shown to bind Gcn4 in vitro. Remarkably, half of the genes in the Pokholok set showed increased expression in our experiment (Fig. 3A, C). We considered it a positive match if at least one of the two genes with a shared promoter (27) was found in our set. Thus, approximately one-third of the genes whose expression increased in response to Snf1 inhibition are predicted to require Gcn4 for their transcriptional activation.

To confirm our microarray data, we analyzed several of the Snf1-repressed genes by northern blot analysis, focusing on those involved in amino acid biosynthesis (Fig. 4). We measured mRNA levels for these genes in snf1Δ cells containing plasmids that express Snf1 (SNF1), Snf1-1132G (snf1-as) or no Snf1 protein (empty vector, snf1Δ). The northern blot analysis confirmed that treatment of the snf1-as strain with 2NM-PP1 induced expression of genes involved in amino acid biosynthesis (compare lanes 4 and 5). The expression of many of these genes was repressed when SNF1 cells were transferred from glucose to
raffinose media, a condition where Snf1 is more active (compare lanes 1 and 2), and increased in snf1Δ cells, relative to SNF1 cells in raffinose media (compare lanes 2 or 4 to lane 3). Interestingly, a few of the genes, notably HIS3, ARG3 and HIS1, showed higher levels of expression in the inhibitor-treated snf1-as strain than in the snf1Δ strain. This suggests that cells may adapt to the absence of Snf1 and shows the power of using the analog-sensitive allele to look at acute effects of inactivating Snf1.

Snf1 represses ARG1 expression in glucose medium. – Our analysis of Gcn4-dependent transcripts showed that in many cases, including the well-studied ARG1 gene, the increase in mRNA levels observed in the snf1-as strain was also detected in a snf1Δ strain grown in raffinose medium (Fig. 4, compare lanes 3 and 5). Therefore, we chose to use snf1Δ strains in the remainder of the experiments presented here to simplify the growth conditions and analysis. We also examined the Snf1-dependent repression of ARG1 in strains grown in the presence of high levels of glucose. Deletion of SNF1 caused a clear increase in ARG1 expression under these conditions (Fig. 5, lanes 1 and 2). Although Snf1 activity is greatly reduced in cells grown in glucose-rich media (28), the residual, low level of Snf1 activity is apparently sufficient to repress ARG1 expression.

Induction of ARG1 in the absence of Snf1 is dependent on the Gcn4 activator. – To determine whether the increase in ARG1 mRNA in the absence of Snf1 was dependent on the Gcn4 activator, we constructed strains lacking SNF1, GCN4 or both genes (Fig. 5). Gen4 is required to generate ARG1 mRNA in snf1Δ strains (lanes 4 and 8), suggesting that derepression of ARG1 in the absence of Snf1 does not bypass the requirement for Gen4. Rather, Snf1 most likely regulates ARG1 transcription at a step prior to activator function.

Snf1 affects Gcn4 protein levels but not GCN4 mRNA levels. – A simple explanation for the induction of ARG1 transcription in snf1Δ strains would be an increase in Gcn4 levels. Although many studies have shown Snf1 to activate transcription, Snf1 is also known to cause repression of some genes, most notably HXT1 (29). We first asked whether Snf1 repressed GCN4 transcript levels. By northern blot analysis, we found that deletion of SNF1 did not affect the level of GCN4 mRNA, regardless of whether cells were grown using glucose or raffinose as a carbon source (Fig. 5). Therefore, the effect of Snf1 on the expression of Gcn4-regulated genes is not due to changes in GCN4 mRNA abundance.

The control of Gcn4 protein levels by regulation of its translation and stability have been well characterized (7). When cells are grown in amino acid rich conditions, translation of Gcn4 is repressed and proteolysis of Gcn4 is enhanced. To determine if Snf1 is involved in regulating Gcn4 translation or stability, we placed three copies of the HA-epitope on the carboxy-terminus of Gcn4 by integrative transformation and measured Gcn4 protein levels by western blot analysis in wild-type and snf1Δ strains. Consistent with an increase in ARG1 transcription, levels of the Gcn4 activator strongly increased in the absence of Snf1, regardless of the carbon source in the medium (Fig. 6A). This increase in Gcn4 levels was observed even though the SC medium used in this experiment was rich in amino acids.

Snf1 regulation of Gcn4 protein abundance depends on the upstream regulatory ORFs. – To investigate whether Snf1 was affecting translation or stabilization of Gcn4, we asked whether Snf1 affected levels of a constitutively expressed form of Gcn4, termed Gcn4c. When the four uORFs in the 5' regulatory region of the GCN4 mRNA are mutated, translation of GCN4 is no longer controlled by the Gcn2 kinase pathway, resulting in constitutively expressed Gcn4 even in amino acid rich media (19). If Snf1 stimulates the degradation of Gcn4, Gcn4c levels should increase in strains lacking SNF1. However, if Snf1 represses GCN4 translation through the uORFs, we would expect that deleting SNF1 would have no effect on Gcn4c protein levels. To examine Gcn4c levels, we placed the 13myc epitope at the C-terminus of Gcn4c (see Experimental Procedures). As shown for Gcn4-HA, Gcn4-13myc levels were also increased in the absence of Snf1 (Fig. 6B, compare lanes 1 and 2). In contrast, Gcn4c-13myc levels did not increase when SNF1 was deleted (Fig. 6B, compare lanes 3 and 4). This result strongly suggests that Snf1 represses the translation of GCN4 in amino acid rich media.

To confirm our findings, we examined the role of Snf1 in regulating the expression of a GCN4-lacZ fusion gene. Plasmids containing GCN4-lacZ
(p180) or GCN4c-lacZ (p227) were transformed into SNF1 and snf1Δ strains, and β-galactosidase assays were performed. We found that snf1Δ strains exhibited increased GCN4-lacZ expression, relative to SNF1 strains, only when the 5' uORFs were present (Fig. 6C).

To control for the possibility that the absence of Snf1 reduces amino acid pools and consequently induces amino acid starvation in our auxotrophic strains, we repeated the ARG1 northern blot and GCN4-lacZ assays in prototrophic strains. Similar to our results with auxotrophs, ARG1 mRNA levels and GCN4-lacZ expression were elevated in the snf1Δ prototroph compared to the SNF1 prototroph (data not shown). These results further support the conclusion that Snf1 represses the translation of GCN4 mRNA.

Snf1 regulation of Gcn4 protein abundance requires other Snf1 complex subunits. Snf1 kinase complex, like AMPK, is composed of the α, or catalytic, subunit Snf1, the γ subunit Snf4, and one of three β subunits, Gal83, Sip1 or Sip2. Previous work has suggested that the β subunits may control substrate selection (11) and regulate the subcellular localization of Snf1 isoforms under different growth conditions (30). To determine whether all three isoforms of Snf1 were competent for repression of GCN4 translation, the plasmid p180 was transformed into strains lacking one, two or all three β subunits, and β-galactosidase assays were performed. A strain lacking all three β subunits lacks Snf1 activity (11) and showed increased GCN4-lacZ expression (Fig. 6D). Analysis of strains expressing only one β subunit showed that the Gal83 and Sip1 isoforms of the Snf1 kinase complex are able to repress GCN4-lacZ expression. Interestingly, the Sip2 isoform is not able to fully repress GCN4-lacZ expression. Thus, two of the three isoforms of the Snf1 kinase complex participate in the repression of GCN4 translation.

The Snf4 (γ) subunit is required for the full activation of the Snf1 kinase (31). We asked whether strains lacking Snf4 would also up-regulate Gcn4 expression, using the GCN4-lacZ reporter plasmid. We found that Snf4 is required to fully repress GCN4 translation, although expression of GCN4-lacZ in snf4Δ cells was not induced to the same levels as in cells lacking Snf1 or all three β subunits. The partial derepression of GCN4-lacZ in the snf4Δ strain is consistent with earlier studies in which deletion of SNF4 produces only a partial defect in invertase induction (11, 31).

Snf1 regulation of Gcn4 protein abundance requires Gcn2 and Gcn20 – GCN4 translation is controlled by activation of the Gcn2 kinase by uncharged tRNAs when amino acid supplies in the cell are reduced. Gcn20, in complex with Gcn1, stimulates Gcn2 activation mediated by uncharged tRNAs (32). To address whether the increase in Gcn4 protein levels seen in the absence of Snf1 requires Gcn2 or Gcn20, we created strains that expressed Gcn4-HA and in which the SNF1, GCN2 or GCN20 genes were deleted. As shown above, deletion of SNF1 increased synthesis of Gcn4-HA (Fig. 7A and B, lane 2). However, when GCN2 or GCN20 was deleted, Gcn4 levels were no longer induced in the absence of Snf1 (lane 3). Deletion of SNF1, GCN2 or GCN20 did not induce any HA-reactive bands in the absence of Gcn4-HA (data not shown). Therefore, induction of Gcn4 in snf1Δ strains requires Gcn2 and Gcn20, indicating that Snf1 control of Gcn4 levels occurs at a step prior to activation of eIF2α by Gcn2 (Fig. 8 and see Discussion). Dependence on Gcn2 and Gcn20 further supports a role for Snf1 in regulating the translation of GCN4 and is not consistent with Snf1 regulating the stability of Gcn4.

DISCUSSION

In this study, we utilized an analog-sensitive version of the Snf1 kinase and microarray analysis to identify genes whose transcription is regulated by Snf1 in S. cerevisiae. Our methodology, in which Snf1 kinase activity was specifically disrupted following its activation, was designed to identify genes repressed by Snf1. Genes involved in amino acid metabolism comprised the largest class of Snf1-repressed genes. These genes are primarily regulated by the Gcn4 transcriptional activator. By investigating the mechanism of Snf1 repression of Gcn4-regulated genes, we uncovered a role for Snf1 in inhibiting GCN4 translation.

Previous studies on Snf1, relying on strains in which SNF1 was deleted, have concentrated on its role as an activator of glucose-repressed genes. Using the chemical inhibition method employed...
here, detection of genes activated by Snf1 would require that the mRNA transcripts from these genes be significantly degraded during the forty-minute incubation with the kinase inhibitor. In contrast, the levels of mRNA transcripts from Snf1-repressed genes would increase during the incubation with inhibitor. In addition, the use of snf1-as strains to rapidly inactivate Snf1 should reveal genes whose regulation may be only transiently affected. Moreover, strains can adapt to the absence of a gene product during growth, and the use of inhibitor-treated snf1-as strains may uncover genes that are more directly regulated by Snf1 when compared with snf1Δ strains. Although our analysis revealed both Snf1-activated and Snf1-repressed genes, we chose to focus on genes that would provide insight into the role of Snf1 as a gene repressor.

A recent microarray study by Young et al. (3) using a snf1Δ strain found that over 400 genes require Snf1 for high expression when cells are grown for six hours in rich media containing low glucose levels (0.05%). While this group noted that Snf1 represses some genes, including ADH1, they did not report the induction of a large number of amino acid biosynthetic genes in the snf1Δ strain. Applying the same cutoff value of a 2.8-fold difference in expression that we used, only 13% of the 117 genes that were up-regulated by snf1Δ in the Young et al. study (3) are classified in the "amino acid and derivative metabolic process" GO-ID (25). Expression of some amino acid biosynthetic genes may be only transiently increased upon Gcn4 activation (33), and thus our protocol may have improved the collective recognition of Gcn4-regulated genes. Nonetheless, in the Young et al. (3) data set, ARG1 mRNA levels were increased approximately twelve-fold in cells lacking Snf1. We also found ARG1 transcript levels to be elevated in snf1Δ strains, here in both glucose and raffinose media. This enabled us to use snf1Δ strains in the remaining analysis of Gcn4 regulation.

During amino acid starvation, Gcn4 levels are governed by both increased mRNA translation and decreased degradation (6, 7, 34). Although we have not directly tested a role for Snf1 in the degradation of Gcn4, our results are most consistent with Snf1 regulating GCN4 translation. First, levels of Gcn4 protein translated from its native mRNA were significantly increased in the absence of Snf1. However, when expression of Gcn4 was rendered insensitive to translation control by mutating the uORFs in the 5' region of GCN4 mRNA, removal of Snf1 had little effect on Gcn4 protein levels. If Snf1 stimulated Gcn4 degradation, we would expect Gcn4 protein levels to increase in a snf1Δ strain regardless of its translation control. Second, expression of a GCN4-lacZ fusion whose translation is controlled by the GCN4 uORFs was also increased in snf1Δ strains. In this construct, the β-galactosidase ORF is fused to the GCN4 ORF following amino acid 55 of Gcn4 (18, 19). This fusion lacks four of the five amino acids that are phosphorylated by the Srb10 kinase in vitro and are required for Gcn4 ubiquitylation in vivo (35). Third, the up-regulation of Gcn4 protein expression in the absence of Snf1 requires two translation control factors, Gcn2 and Gcn20, proteins not known to play a role in Gcn4 degradation. Taken together, the data suggest a role for Snf1 in controlling GCN4 mRNA translation under amino acid-rich conditions.

Although we detected Snf1 as a repressor of GCN4 translation in media rich in amino acids, others have found that Snf1 or Snf1-activating conditions can induce GCN4 translation (36) or Gcn4-dependent transcription (37) in media supplemented with only the amino acids necessary to complement strain auxotrophies. Yang et al. (36) found that GCN4-lacZ translation was induced in cells starved for glucose for six hours. Starving for glucose should induce Snf1 kinase activity (28, 38), and our data would predict that GCN4 translation would be further repressed. However, minimal amino acid media may cause an initial nutritional stress to cells such that further stress, such as low glucose conditions, causes the induction of Gcn4 protein. This would be consistent with the work by Liu et al. (37), who found that Snf1 was required for transcription of the Gcn4-dependent HIS3 gene in cells treated with 40 mM 3-amino-1,2,4-triazole and incubated at 37°C. In contrast, we have found that Snf1 represses GCN4 translation under growth conditions where amino acids and sugar are plentiful. Interestingly, growth of prototrophic snf1Δ or snf4Δ strains in low glucose in a chemostat also induced expression of proteins involved in amino acid metabolism (39). Although our results were first discovered in cells grown in raffinose, we found similar results in glucose media. This indicates that
Snf1 is active, to some extent, in cells utilizing glucose as a carbon source, a condition typically thought to repress Snf1 activity. A more accurate description is that Snf1 activity is reduced, not absent, in the presence of high glucose. Snf1 activity in medium containing high glucose levels is evident when one compares SNF1+ and snf1Δ cells for invertase activity (11), Mig1 phosphorylation (11), inositol auxotrophy (40) or pseudohyphal growth (41). Collectively, our results and others’ raise the interesting possibility that the Snf1 kinase may act as a regulatory switch in a signal transduction pathway that relays nutrient availability information to the translation machinery.

Gcn2 kinase stimulates GCN4 translation by phosphorylating eIF2α (6, 34, 42) (Fig. 8). In cells growing in media with high levels of amino acids, Gcn2 kinase is maintained in an inactive state by the reduced levels of uncharged tRNAs and by TOR-dependent phosphorylation of the serine at amino acid 577 (S577) (6). When cells are starved for amino acids, Gcn2 kinase is activated by binding to the more abundant uncharged tRNAs, in a manner stimulated by the Gcn1/Gcn20 complex, but not requiring the dephosphorylation of S577 of Gcn2 (43). Under conditions that inactivate the TOR pathway, such as rapamycin treatment, a Tap42/PP2A or Tap42/Sit4 phosphatase complex can dephosphorylate S577 of Gcn2 (43). This dephosphorylation increases the affinity of Gcn2 for uncharged tRNAs, activating Gcn2 kinase even in amino acid replete conditions. We show here that induction of GCN4 translation in the absence of Snf1 requires Gcn2 and Gcn20.

The mechanism by which Snf1 represses Gcn4 translation in nutrient-rich conditions is unknown. Snf1 could regulate the activity of Gcn2 directly or indirectly by stimulating phosphorylation of S577 (Fig. 8). Consistent with this possibility, we have previously shown that the Sit4 phosphatase and the Snf1 kinase have opposing functions in regulating transcription of the INO1 gene (23). Our results are also consistent with a mechanism in which Snf1 inhibits the activity of the Gcn1/Gcn20 complex. These mechanisms may not be independent because a previous genetic analysis of rapamycin sensitivity indicated that both Gcn2 and Gcn20 function downstream of TOR kinase (44). Regardless of the exact mechanism, the dependency of Gcn4 translational induction on Gcn2 suggested that the levels of phosphorylated eIF2α should be elevated in snf1Δ strains. Unexpectedly, our initial assays have revealed similar eIF2α-P levels in SNF1 and snf1Δ strains grown in SC media (data not shown). This finding may indicate that Snf1 impacts Gcn2 and/or eIF2α activity by both positive and negative mechanisms, as has been proposed for Sit4 (6), negating a detectable effect on eIF2α-P levels in the absence of Snf1. Determining the direct target of the Snf1 kinase involved in GCN4 translation will distinguish among possible mechanisms and further elucidate the cross-talk in pathways that regulate glucose repression and amino acid biosynthesis.

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| Strain   | Genotype                                                                 |
|----------|--------------------------------------------------------------------------|
| FY1193   | MAT α hisΔ200 leuΔ1 ura3-52 trpΔ63 snf1Δ10                                |
| KY1200   | MAT a hisΔ200 leuΔ1 ura3-52 trpΔ63                                       |
| MSY520   | MAT a hisΔ200 leuΔ1 ura3-52 trpΔ63 sip2Δ::HIS3                           |
| MSY522   | MAT a hisΔ200 leuΔ1 ura3-52 trpΔ63 gal83Δ::HIS3                           |
| MSY528   | MAT a hisΔ200 leuΔ1 ura3-52 trpΔ63 sip1Δ::HIS3                           |
| MSY543   | MAT a hisΔ200 leuΔ1 ura3-52 trpΔ63 gal83Δ::HIS3 sip2Δ::HIS3             |
| MSY544   | MAT a hisΔ200 leuΔ1 ura3-52 trpΔ63 sip1Δ::HIS3 sip2Δ::HIS3             |
| MSY552   | MAT a hisΔ200 leuΔ1 ura3-52 trpΔ63 gal83Δ::HIS3 sip1Δ::HIS3             |
| MSY557   | MAT a hisΔ200 leuΔ1 ura3-52 trpΔ63 gal83Δ::HIS3 sip1Δ::HIS3 sip2Δ::HIS3 |
| MSY848   | MAT a hisΔ200 leuΔ1 ura3-52 trpΔ63 snf4Δ1                               |
| PY133    | MAT a hisΔ200 ura3-52 snf1Δ10                                            |
| PY855    | MAT a hisΔ200 ura3-52 snf1Δ10 [pSNF1-316=CEN/ARS, URA3, HA-SNF1]       |
| PY856    | MAT a hisΔ200 ura3-52 snf1Δ10 [pSNF1-1132G-316=CEN/ARS, URA3, HA-snf1-as]|
| PY1003   | MAT a hisΔ200 leuΔ1 ura3-52 trpΔ63 gcn4Δ::KANMX4                        |
| PY1008   | MAT a hisΔ200 leuΔ1 ura3-52 trpΔ63 snf1Δ10 gcn4Δ::KANMX4               |
| PY1012   | MAT a hisΔ200 leuΔ1 ura3-52 trpΔ63 snf1Δ10                               |
| PY1017   | MAT α hisΔ200 leuΔ1 ura3-52 trpΔ63 GCN4-13myc::KANMX6                  |
| PY1064   | MAT a hisΔ200 leuΔ1 ura3-52 trpΔ63 snf1Δ10 GCN4-HA::KANMX6           |
| PY1070   | MAT a hisΔ200 leuΔ1 ura3-52 trpΔ63 GCN4-HA::KANMX6                     |
| PY1094   | MAT α leuΔ1 ura3-52 trpΔ63                                              |
| PY1095   | MAT α leuΔ1 ura3-52 trpΔ63 snf1Δ10                                      |
| PY1118   | MAT a hisΔ200 leuΔ1 ura3-52 trpΔ63 gen2Δ::KANMX6                        |
| PY1139   | MAT a hisΔ200 leuΔ1 ura3-52 trpΔ63 snf1Δ10 gen2Δ::KANMX6               |
| PY1140   | MAT a hisΔ200 leuΔ1 ura3-52 trpΔ63 gen2Δ::KANMX6 GCN4-HA::KANMX6        |
| PY1141   | MAT a hisΔ200 leuΔ1 ura3-52 trpΔ63 snf1Δ10 gen2Δ::KANMX6 GCN4-HA::KANMX6|
| PY1175   | MAT a hisΔ200 leuΔ1 ura3-52 trpΔ63 snf1Δ10 gen20Δ::KANMX4              |
| PY1195   | MAT a hisΔ200 leuΔ1 ura3-52 trpΔ63 snf1Δ10 gen20Δ::KANMX4 GCN4-HA::KANMX6|
| Strain  | Genetic Information                                                                 |
|---------|-------------------------------------------------------------------------------------|
| PY1201  | *MATα his3Δ200 leu2Δ1 ura3-52 trp1Δ63 gcn20Δ::KANMX4 GCN4-HA::KANMX6*              |
| PY1207  | *MATα his3Δ200 leu2Δ1 ura3-52 trp1Δ63 gcn20Δ::KANMX4*                              |
FIGURE 1. Analog-sensitive allele of Snf1. A, Halo assay of cells expressing wild-type Snf1 (bottom) or Snf1-I132G (top). Cells were spread on agar plates with sucrose as the carbon source. Sterile paper discs were spotted with either Me$_2$SO or inhibitor compounds dissolved in Me$_2$SO as indicated. B, Structure of 2NM-PP1. C, Growth curve of PY855 (Snf1) or PY856 (Snf1-I132G) cells grown in SC medium lacking uracil with 2% raffinose and 0.05% glucose as the carbon sources. Cells expressing either wild-type Snf1 (open symbols) or Snf1-I132G (filled symbols) were grown to OD$_{600}$ of 0.3, at which time Me$_2$SO (circles) or 2NM-PP1 dissolved in Me$_2$SO (squares) was added to a final concentration of 25 µM.
FIGURE 2. **2NM-PP1 treatment of strains expressing Snf1-I132G reduces SUC2 transcription.**
Northern blot analysis of total yeast RNA (10 µg per lane) prepared from PY855 (Snf1) or PY856 (Snf1-I132G) cells is shown. Filters were probed sequentially with sequences from the **SUC2** and **ACT1** genes as shown. Cells were grown in 2% glucose, then shifted to medium containing 2% raffinose and 0.05% glucose for two hours. Cultures were divided and treated with Me$_2$SO or 2% glucose as controls or with varying concentrations of 2NM-PP1 dissolved in Me$_2$SO for the indicated times.
FIGURE 3. **Microarray analysis of Snf1-I132G.** A, Scatter plot of mRNA abundance in cells expressing Snf1 or Snf1-I132G and treated with 2NM-PP1. FY1193 (snf1Δ) cells expressing either wild-type Snf1 (pSNF1-316) or Snf1-I132G (pSNF1-I132G-316) were transferred to SC-Ura medium containing 2% raffinose and 0.05% glucose for 2 hr and incubated in the presence of 2NM-PP1 for 40 min. RNA was processed for microarray analysis (see Experimental Procedures). Mean log2 values from two independent microarray data sets were plotted with wild-type Snf1 on the x-axis and Snf1-I132G on the y-axis. Genes differentially expressed in the absence of Snf1 kinase activity are shown above and below the lines indicating mean log2 differences of 1.5. Genes whose expression is Gcn4-dependent (27) are shown in dark grey. B, Scaled Venn diagram showing intersection of genes up-regulated in Snf1-I132G cells treated with inhibitor and genes down-regulated in the absence of Gcn4 (26). C, Scaled Venn diagram showing intersection of genes upregulated in Snf1-I132G cells treated with inhibitor and genes predicted to be direct targets of Gcn4 (27).
FIGURE 4. Snf1 kinase activity inhibits expression of many Gcn4-dependent genes. Total RNA was prepared from cells grown in 2% glucose (G) or shifted to 2% raffinose and 0.05% glucose (R) for 160 minutes. FY1193 cells were transformed with pRS316 (snf1Δ), pSNF1-316 (SNF1) or pSNF1-I132G-316 (snf1-as). For the samples shown in lanes 4 and 5, cells were treated with 25 µM 2NM-PP1 dissolved in Me2SO for 40 minutes prior to RNA extraction. RNA was detected by northern blot analysis with the probes indicated on the right of each panel. Grouped data were obtained using successive hybridizations to the same filter. The data for ARGI were all from the same scanned film, with the lanes rearranged for consistency with the other data presented here. SCR1 encodes the RNA subunit of the signal recognition particle and was used as an RNA loading control.
FIGURE 5. **ARG1 expression in the absence of Snf1 requires Gcn4 activity.** Total RNA was extracted from KY1200 (WT), PY1012 (snf1Δ), PY1003 (gcn4Δ) or PY1008 (snf1Δ gcn4Δ) cells. Cells were grown in SC complete medium containing 2% glucose (Glu), and a portion of the culture was washed and transferred to SC complete medium containing 2% raffinose and 0.05% glucose (Raf) for 160 min. RNAs were detected by northern blot analysis with probes for **ARG1**, **GCN4** or **SCR1** as shown. **SCR1** RNA levels serve as a loading control.
FIGURE 6. Snf1-mediated regulation of Gcn4 protein levels requires the small open reading frames in the GCN4 mRNA 5' leader region. A, Wild-type SNF1 (PY1070, +) and snf1Δ (PY1064, Δ) cells expressing HA-tagged Gcn4 were grown in SC complete medium containing 2% glucose (Glu) and a portion of the culture was washed and transferred to SC complete medium containing 2% raffinose and 0.05% glucose (Raf) for 160 minutes. Protein extracts were prepared and analyzed by western blotting with antibodies directed against HA or Sse1. B, Wild-type SNF1 (PY1003) or snf1Δ (PY1008) cells were transformed with plasmids pPS128 (Gcn4-13myc, lanes 1 and 2) or pPS127 (Gcn4c-13myc, lanes 3 and 4). Cells were grown in SC-Ura medium containing 2% glucose. Protein extracts were prepared and analyzed by western blotting with antibodies directed against the c-myc epitope or Sse1. In an average of five experiments, normalized Gcn4c-13myc levels were only 1.14-fold higher in snf1Δ cells compared to SNF1 cells. C, Wild-type SNF1 (PY1070) and snf1Δ (PY1064) cells were transformed with plasmids encoding β-galactosidase fused after amino acid 55 of the GCN4 open reading frame. The GCN4c-lacZ plasmid contains mutations that disrupt the small open reading frames in the GCN4 mRNA 5' region. Extracts were prepared from cells grown in glucose medium and assayed for β-galactosidase activity. Error bars show one standard error of the mean. D, WT SNF1 (PY1094), snf1Δ (PY1095), gal83Δ (MSY522), sip1Δ (MSY528), sip2Δ (MSY520), gal83Δ sip1Δ (MSY552), gal83Δ sip2Δ (MSY543), sip1Δ sip2Δ (MSY544), gal83Δ sip1Δ sip2Δ (MSY557), and snf4Δ (MSY848) cells were transformed with the GCN4-lacZ plasmid. To control for the histidine prototrophy of the strains lacking the β subunits, SNF1 HIS3 and snf1Δ HIS3 strains were used in this analysis. Extracts were prepared and assayed as described in C. The source of the higher β-galactosidase levels in the absence of all three β subunits is unknown but could be due to an indirect effect of expressing three copies of HIS3, a Gcn4-regulated gene, in the absence of a functional Snf1 complex.
FIGURE 7. Snf1 regulation of Gcn4 abundance requires Gcn2 and Gcn20. A and B, Cells expressing HA-tagged Gcn4 protein, in combination with either wild-type (+) or complete deletions (Δ) of the SNF1, GCN2 or GCN20 genes as shown, were grown in SC-Ura medium containing 2% glucose. Protein extracts were prepared and analyzed by western blotting with antibodies against either the HA epitope or Sse1.
FIGURE 8. Model for Snf1 inhibition of GCN4 translation. Gcn2 kinase is stimulated when its HisRS tRNA synthetase-like domain is bound by uncharged tRNA. Stimulation by the HisRS domain is accentuated by the Gcn1/20 complex and attenuated by phosphorylation of Gen2 at Ser577 (6, 45). The TOR kinase pathway inhibits Gcn2 by promoting phosphorylation of Ser577. Dephosphorylation of Ser577 by the PP2A and/or Sit4 phosphatase occurs when TOR is inhibited by rapamycin (43). Snf1 may inhibit the activation of Gcn2 by either promoting phosphorylation of S577 (directly or indirectly through the TOR pathway) or the inhibition of PP2A or Sit4 phosphatase. An alternative but not mutually exclusive model is that Snf1 inhibits Gcn2-dependent translation of Gcn4 by inhibiting the Gcn1/20 complex.
A chemical-genomics study identifies Snf1 as a repressor of Gcn4 translation
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