

# A Chemical Genomics Study Identifies Snf1 as a Repressor of GCN4 Translation\*<sup>§</sup>

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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)<sup>2</sup> 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 *Saccharomyces cerevisiae* *SNF1* gene that could be rapidly inactivated upon exposure to a pyrazolopyrimidine inhibitor (4). In contrast to using an *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 eIF2 $\alpha$ . Phosphorylation of eIF2 $\alpha$  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 cross-talk 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  $A_{600}$  of 0.1. Cultures were grown until the  $A_{600}$  reached 0.3, divided, and incubated with either dimethyl sulfoxide (Me<sub>2</sub>SO) or 25  $\mu$ M 2NM-PP1 dissolved in Me<sub>2</sub>SO, and  $A_{600}$  values were recorded every 30 min.

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<sup>§</sup> The on-line version of this article (available at <http://www.jbc.org>) contains supplemental Table 1.

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<sup>2</sup> The abbreviations used are: AMPK, AMP-activated protein kinase; TOR, target of rapamycin; uORF, upstream open reading frame; HA, hemagglutinin; GO, gene ontology.

**TABLE 1**  
**S. cerevisiae strains**

Strain	Genotype
FY1193	MAT $\alpha$ his3 $\Delta$ 200 leu2 $\Delta$ 1 ura3-52 trp1 $\Delta$ 63 snf1 $\Delta$ 10
KY1200	MAT $\alpha$ his3 $\Delta$ 200 leu2 $\Delta$ 1 ura3-52 trp1 $\Delta$ 63
MSY520	MAT $\alpha$ his3 $\Delta$ 200 leu2 $\Delta$ 1 ura3-52 trp1 $\Delta$ 63 sip2 $\Delta$ ::HIS3
MSY522	MAT $\alpha$ his3 $\Delta$ 200 leu2 $\Delta$ 1 ura3-52 trp1 $\Delta$ 63 gal83 $\Delta$ ::HIS3
MSY528	MAT $\alpha$ his3 $\Delta$ 200 leu2 $\Delta$ 1 ura3-52 trp1 $\Delta$ 63 sip1 $\Delta$ ::HIS3
MSY543	MAT $\alpha$ his3 $\Delta$ 200 leu2 $\Delta$ 1 ura3-52 trp1 $\Delta$ 63 gal83 $\Delta$ ::HIS3 sip2 $\Delta$ ::HIS3
MSY544	MAT $\alpha$ his3 $\Delta$ 200 leu2 $\Delta$ 1 ura3-52 trp1 $\Delta$ 63 sip1 $\Delta$ ::HIS3 sip2 $\Delta$ ::HIS3
MSY552	MAT $\alpha$ his3 $\Delta$ 200 leu2 $\Delta$ 1 ura3-52 trp1 $\Delta$ 63 gal83 $\Delta$ ::HIS3 sip1 $\Delta$ ::HIS3
MSY557	MAT $\alpha$ his3 $\Delta$ 200 leu2 $\Delta$ 1 ura3-52 trp1 $\Delta$ 63 gal83 $\Delta$ ::HIS3 sip1 $\Delta$ ::HIS3 sip2 $\Delta$ ::HIS3
MSY848	MAT $\alpha$ his3 $\Delta$ 200 leu2 $\Delta$ 1 ura3-52 trp1 $\Delta$ 63 snf4 $\Delta$ 1
PY133	MAT $\alpha$ his3 $\Delta$ 200 ura3-52 snf1 $\Delta$ 10
PY855	MAT $\alpha$ his3 $\Delta$ 200 ura3-52 snf1 $\Delta$ 10 [pSNF1-316 = CEN/ARS, URA3, HA-SNF1]
PY856	MAT $\alpha$ his3 $\Delta$ 200 ura3-52 snf1 $\Delta$ 10 [pSNF1-1132G-316 = CEN/ARS, URA3, HA-snf1-as]
PY1003	MAT $\alpha$ his3 $\Delta$ 200 leu2 $\Delta$ 1 ura3-52 trp1 $\Delta$ 63 gcn4 $\Delta$ ::KANMX4
PY1008	MAT $\alpha$ his3 $\Delta$ 200 leu2 $\Delta$ 1 ura3-52 trp1 $\Delta$ 63 snf1 $\Delta$ 10 gcn4 $\Delta$ ::KANMX4
PY1012	MAT $\alpha$ his3 $\Delta$ 200 leu2 $\Delta$ 1 ura3-52 trp1 $\Delta$ 63 snf1 $\Delta$ 10
PY1017	MAT $\alpha$ his3 $\Delta$ 200 leu2 $\Delta$ 1 ura3-52 trp1 $\Delta$ 63 GCN4-13myc::KANMX6
PY1064	MAT $\alpha$ his3 $\Delta$ 200 leu2 $\Delta$ 1 ura3-52 trp1 $\Delta$ 63 snf1 $\Delta$ 10 GCN4-HA::KANMX6
PY1070	MAT $\alpha$ his3 $\Delta$ 200 leu2 $\Delta$ 1 ura3-52 trp1 $\Delta$ 63 GCN4-HA::KANMX6
PY1094	MAT $\alpha$ leu2 $\Delta$ 1 ura3-52 trp1 $\Delta$ 63
PY1095	MAT $\alpha$ leu2 $\Delta$ 1 ura3-52 trp1 $\Delta$ 63 snf1 $\Delta$ 10
PY1118	MAT $\alpha$ his3 $\Delta$ 200 leu2 $\Delta$ 1 ura3-52 trp1 $\Delta$ 63 gcn2 $\Delta$ ::KANMX6
PY1139	MAT $\alpha$ his3 $\Delta$ 200 leu2 $\Delta$ 1 ura3-52 trp1 $\Delta$ 63 snf1 $\Delta$ 10 gcn2 $\Delta$ ::KANMX6
PY1140	MAT $\alpha$ his3 $\Delta$ 200 leu2 $\Delta$ 1 ura3-52 trp1 $\Delta$ 63 gcn2 $\Delta$ ::KANMX6 GCN4-HA::KANMX6
PY1141	MAT $\alpha$ his3 $\Delta$ 200 leu2 $\Delta$ 1 ura3-52 trp1 $\Delta$ 63 snf1 $\Delta$ 10 gcn2 $\Delta$ ::KANMX6 GCN4-HA::KANMX6
PY1175	MAT $\alpha$ his3 $\Delta$ 200 leu2 $\Delta$ 1 ura3-52 trp1 $\Delta$ 63 snf1 $\Delta$ 10 gcn20 $\Delta$ ::KANMX4
PY1195	MAT $\alpha$ his3 $\Delta$ 200 leu2 $\Delta$ 1 ura3-52 trp1 $\Delta$ 63 snf1 $\Delta$ 10 gcn20 $\Delta$ ::KANMX4 GCN4-HA::KANMX6
PY1201	MAT $\alpha$ his3 $\Delta$ 200 leu2 $\Delta$ 1 ura3-52 trp1 $\Delta$ 63 gcn20 $\Delta$ ::KANMX4 GCN4-HA::KANMX6
PY1207	MAT $\alpha$ his3 $\Delta$ 200 leu2 $\Delta$ 1 ura3-52 trp1 $\Delta$ 63 gcn20 $\Delta$ ::KANMX4

**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*<sup>+</sup> derivative of S288C (10). FY1193 was obtained from Fred Winston. MSY strains have been described previously (11). KY and PY strains were constructed by genetic mating and tetrad dissection or integrative transformations. To generate strains containing the *gcn4 $\Delta$ ::KANMX4* mutation or the *gcn20 $\Delta$ ::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 *snf1 $\Delta$*  and processed for tetrad analysis. The *GCN4-HA::KANMX6* and *gcn2 $\Delta$ ::KANMX4* strains were also constructed by PCR amplification of the appropriate fragments (13, 14) and transformation into diploid strains heterozygous for *snf1 $\Delta$* . 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 QuikChange 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 AflII and XbaI, 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-polyacrylamide 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 AflII-XbaI 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 described previously (18, 19). Oligonucleotide and plasmid sequences are available upon request.

**Yeast Microarray Analysis**—Yeast cultures were grown in triplicate to an  $A_{600}$  of 0.8–1.0 at 30 °C in SC-Ura medium containing 2% glucose. Cultures were shifted to medium containing 2% raffinose and 0.05% glucose for 2 h. The drug 2NM-PP1 was added to cultures to a final concentration of 25  $\mu$ M 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  $\mu$ g 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  $\mu$ g) 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 here have been deposited in NCBI Gene Expression Omnibus (20) and are accessible through GEO Series accession number GSE12061 (www.ncbi.nlm.nih.



gov). In addition, normalized values are given in supplemental 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 Applied Science) and 10 mM NaF, and processed as described (23), except that 50  $\mu$ g of total extract was separated on an SDS-10% polyacrylamide gel. The HA epitope antibody (Roche Applied Science) 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.

**$\beta$ -Galactosidase Assays**—Extract preparations,  $\beta$ -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 Ile-132 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. 1A 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 with 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 *ACT1* mRNA upon shifting cells from glucose to raffinose (Fig. 2, lane 2). The addition of the inhibitor 2NM-PP1 did not affect the induction of *SUC2* mRNA in wild-type cells (Fig. 2, lanes 7 and 8). In the absence of 2NM-PP1, induction of *SUC2* mRNA in the Snf1-I132G strain (Fig. 2, lane 10) was indistinguishable from induction in the wild-type strain (Fig. 2, lane 2). However, addition of 2NM-PP1 to raffinose-grown Snf1-I132G cells returned *SUC2* mRNA levels back to the uninduced state (Fig. 2, lanes 15–22). A titration of the inhibitor showed that 25  $\mu$ M 2NM-PP1 was sufficient to abrogate Snf1-I132G 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  $\mu$ M 2NM-PP1 for 40 min was used to inhibit the activity of Snf1-I132G in subsequent experiments. Taken together, our data show that the combination of the Snf1-I132G 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-I132G 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 2 h 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-as* strain treated with 2NM-PP1 compared with the *SNF1* strain treated with 2NM-PP1 (Fig. 3A and supplemental Table 1). These genes were categorized by their gene ontology (GO) annotation to determine whether any biological processes were enriched in this set of genes (25). Remarkably, 35% ( $p$  value of  $<1 \times 10^{-42}$ ) 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

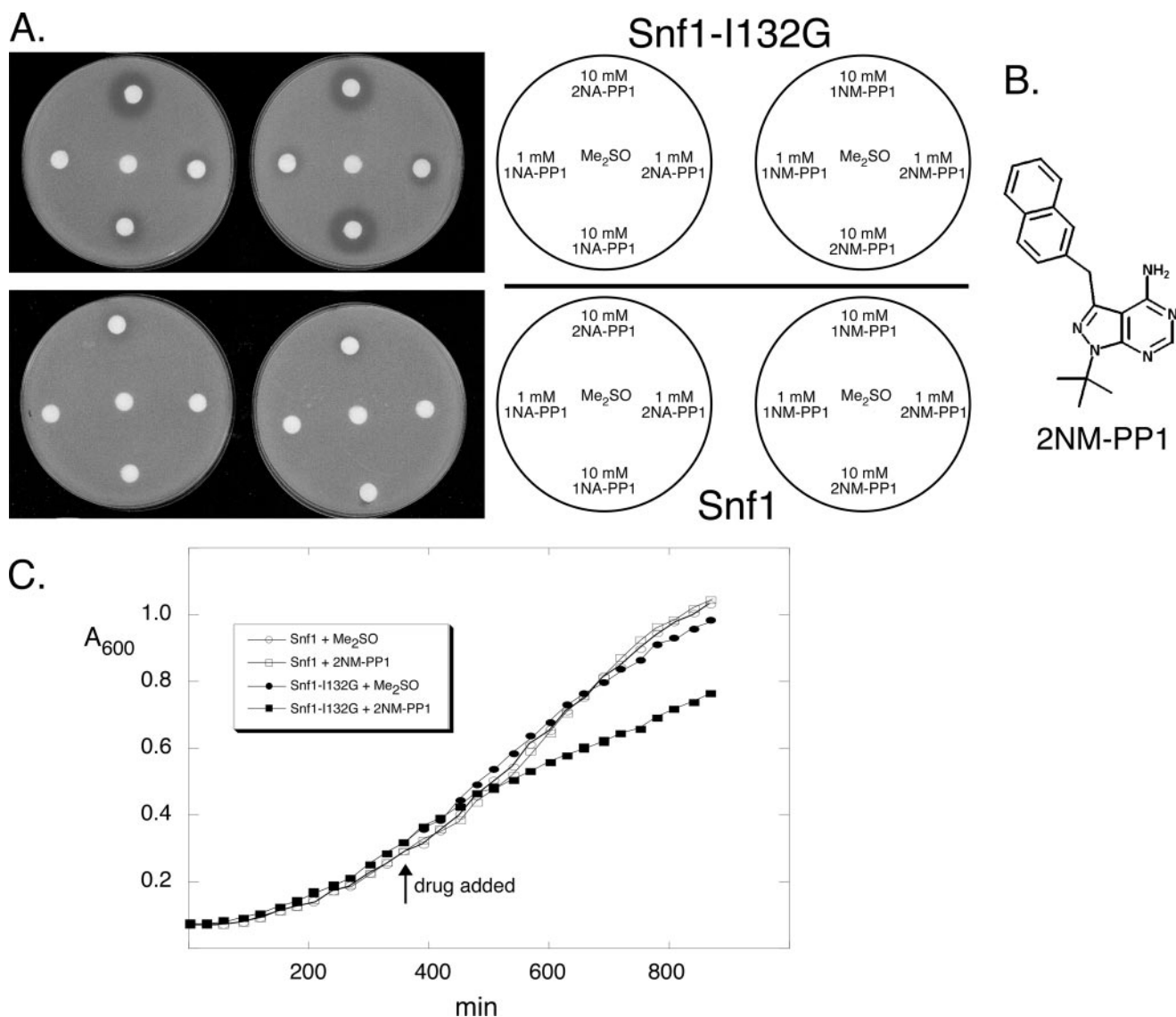


FIGURE 1. **Analog-sensitive allele of Snf1.** A, halo assay of cells expressing wild-type Snf1 (bottom) or Snf1-I132G (top). Cells were spread on agar plates with sucrose as the carbon source. Sterile paper disks were spotted with either Me<sub>2</sub>SO or inhibitor compounds dissolved in Me<sub>2</sub>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 an A<sub>600</sub> of 0.3, at which time Me<sub>2</sub>SO (circles) or 2NM-PP1 dissolved in Me<sub>2</sub>SO (squares) was added to a final concentration of 25 μM.

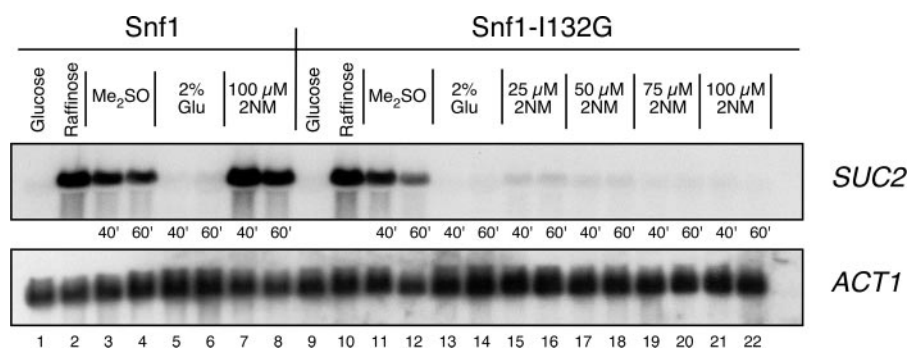
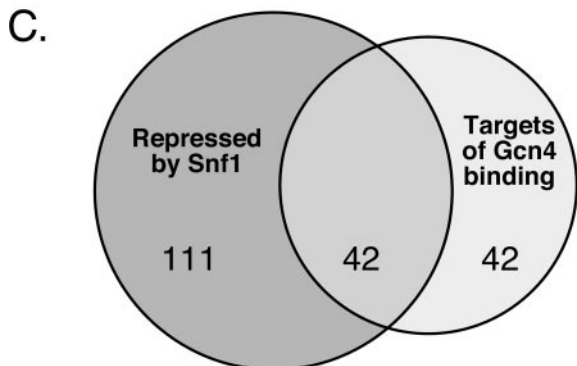
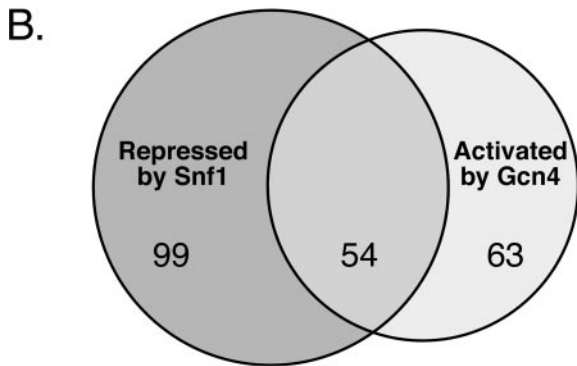
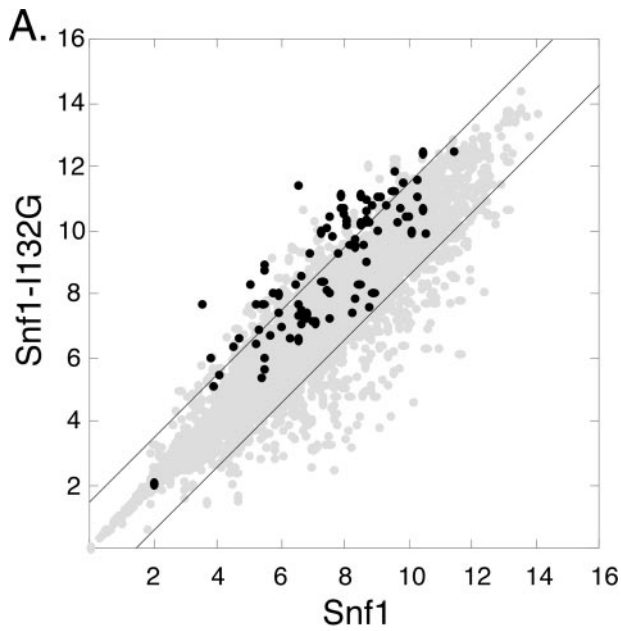
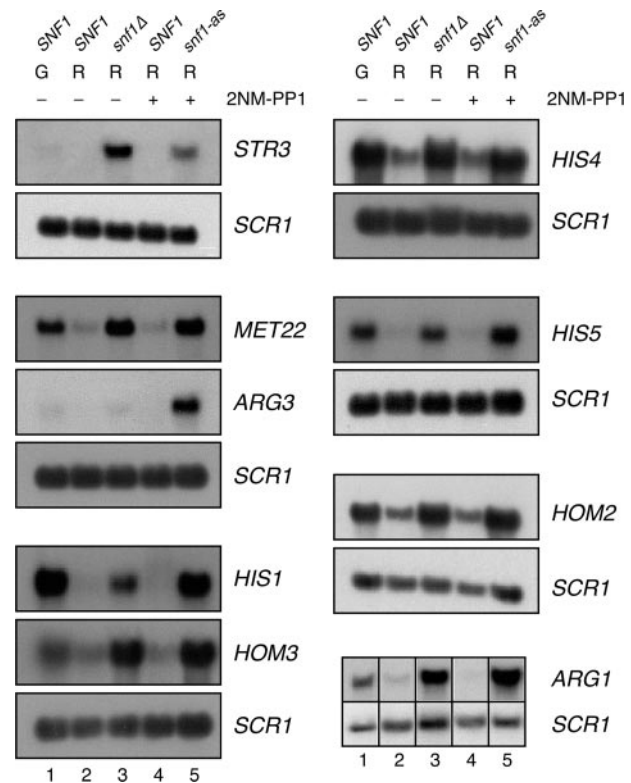


FIGURE 2. **2NM-PP1 treatment of strains expressing Snf1-I132G reduces SUC2 transcription.** Northern blot analysis of total yeast RNA (10 μg per lane) prepared from PY855 (Snf1) or PY856 (Snf1-I132G) cells is shown. Filters were probed sequentially with sequences from the *SUC2* and *ACT1* genes as shown. Cells were grown in 2% glucose and then shifted to medium containing 2% raffinose and 0.05% glucose for 2 h. Cultures were divided and treated with Me<sub>2</sub>SO or 2% glucose as controls or with varying concentrations of 2NM-PP1 dissolved in Me<sub>2</sub>SO for the indicated times.

deleted (26). 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 have 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 starva-



**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 h and incubated in the presence of 2NM-PP1 for 40 min. RNA was processed for microarray analysis (see “Experimental Procedures”). Mean  $\log_2$  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  $\log_2$  differences of 1.5. Genes whose expression is Gcn4-dependent (27) are shown in dark gray. 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 up-regulated in Snf1-I132G cells treated with inhibitor and genes predicted to be direct targets of Gcn4 (27).

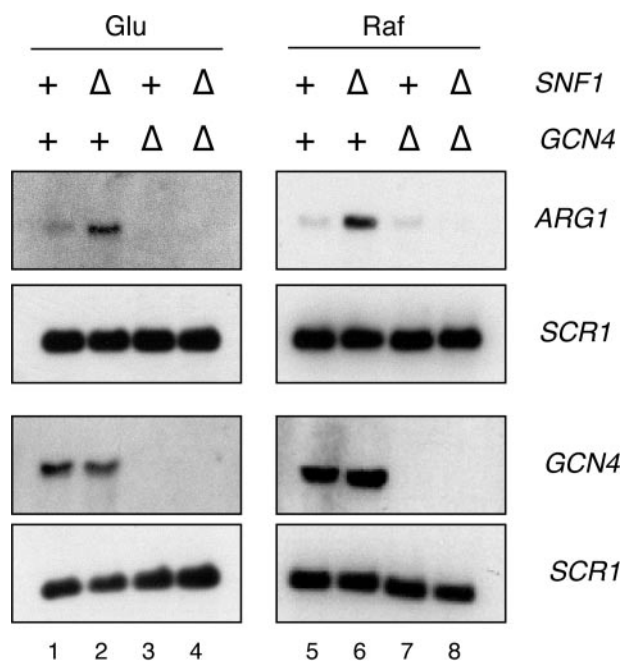


**FIGURE 4. Snf1 kinase activity inhibits expression of many Gcn4-dependent genes.** Total RNA was prepared from cells grown in 2% glucose (G) or shifted to 2% raffinose and 0.05% glucose (R) for 160 min. FY1193 cells were transformed with pRS316 (*snf1Δ*), pSNF1-316 (*SNF1*), or pSNF1-I132G-316 (*snf1-as*). For the samples shown in lanes 4 and 5, cells were treated with 25  $\mu$ M 2NM-PP1 dissolved in Me<sub>2</sub>SO for 40 min prior to RNA extraction. RNA was detected by Northern blot analysis with the probes indicated to the right of each panel. Grouped data were obtained using successive hybridizations to the same filter. The data for ARG1 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.

tion; 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. 3, A and 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-I132G (*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 (Fig. 4, 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 (Fig. 4, 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 *HIS5*,





**FIGURE 5. ARG1 expression in the absence of Snf1 requires Gcn4 activity.** Total RNA was extracted from KY1200 (wild type), 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.

*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 an *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). Gcn4 is required to generate *ARG1* mRNA in *snf1Δ* strains (Fig. 5, lanes 4 and 8), suggesting that derepression of *ARG1* in the absence of Snf1 does not bypass the requirement for Gcn4. Rather, Snf1 most likely regulates *ARG1* transcription at a step prior to activator function.

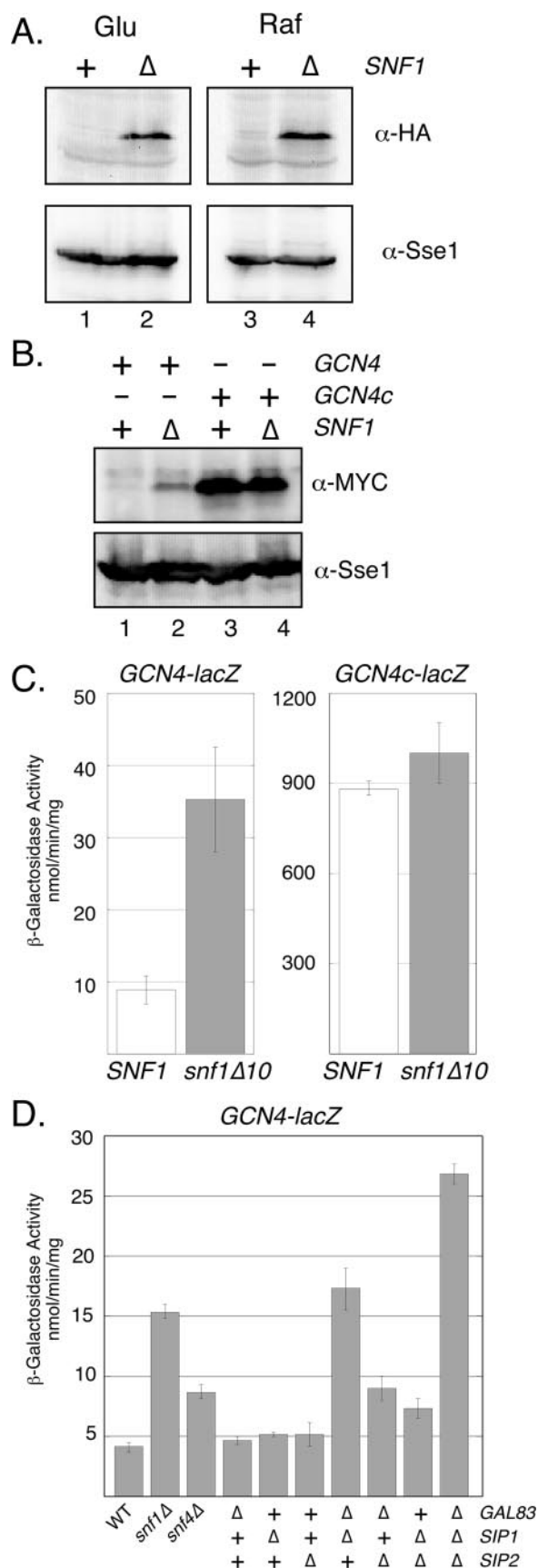
**Snf1 Affects Gcn4 Protein Levels but Not GCN4 mRNA Levels**—A simple explanation for the induction of *ARG1* transcription in *snf1Δ* strains would be an increase in Gcn4 levels. Although many studies have shown Snf1 to activate transcription, Snf1 is also known to cause repression of some genes, most 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 because of changes in *GCN4* mRNA abundance.

The control of Gcn4 protein levels by regulation of its translation and stability has 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 whether Snf1 is involved in regulating Gcn4 translation or stability, we placed three copies of the HA epitope on the C 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  $\beta$ -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 com-



**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 *snf1*Δ (PY1064, Δ) cells expressing HA-tagged

pared with the *SNF1* prototroph (data not shown). These results further support the conclusion that Snf1 represses the translation of *GCN4* mRNA.

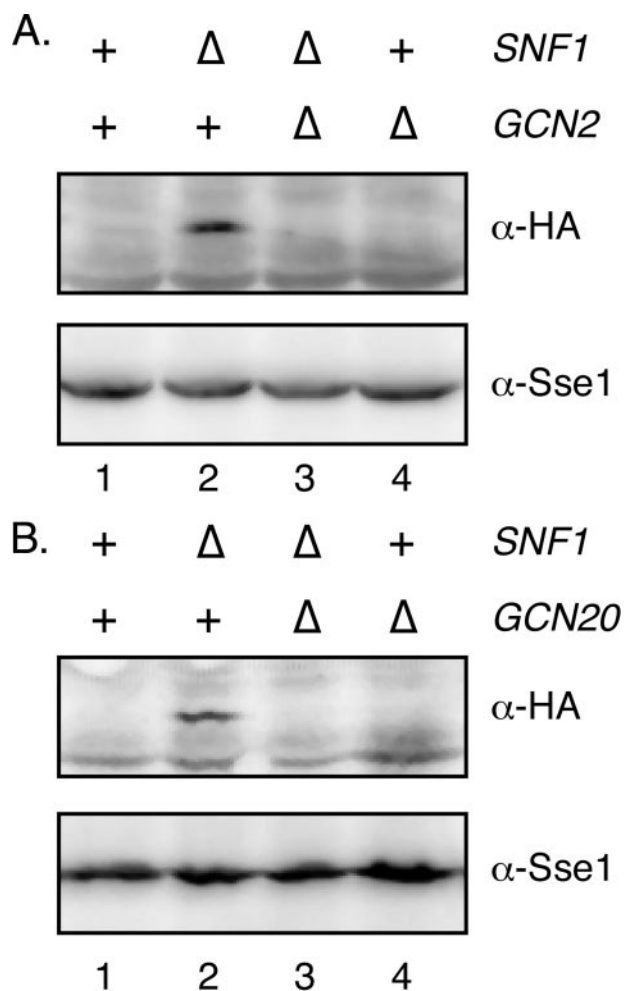
*Snf1* Regulation of *Gcn4* Protein Abundance Requires Other *Snf1* Complex Subunits—Snf1 kinase complex, like AMPK, is composed of the α, or catalytic, subunit Snf1, the γ subunit Snf4, and one of three β subunits, Gal83, Sip1, or Sip2. Previous work has suggested that the β subunits may control substrate 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 *snf4*Δ 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 *snf4*Δ 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

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 min. Protein extracts were prepared and analyzed by Western blotting with antibodies directed against HA or Sse1. *B*, wild-type *SNF1* (PY1003) or *snf1*Δ (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 *snf1*Δ cells compared with *SNF1* cells. *C*, wild-type *SNF1* (PY1070) and *snf1*Δ (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 mean ± 1 S.E. *D*, wild-type (WT) *SNF1* (PY1094), *snf1*Δ (PY1095), *gal83*Δ (MSY522), *sip1*Δ (MSY528), *sip2*Δ (MSY520), *gal83*Δ *sip1*Δ (MSY552), *gal83*Δ *sip2*Δ (MSY543), *sip1*Δ *sip2*Δ (MSY544), *gal83*Δ *sip1*Δ *sip2*Δ (MSY557), and *snf4*Δ (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 *snf1*Δ *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.

## Snf1 Regulation of Gcn4

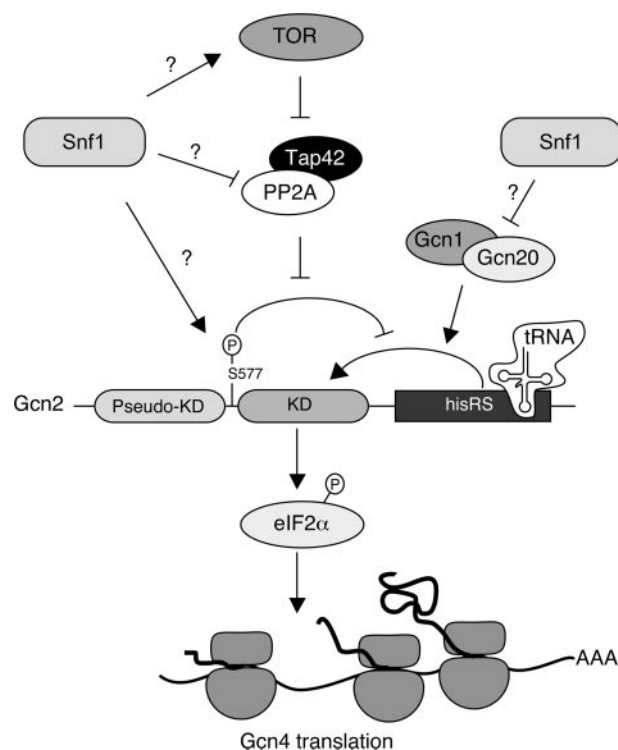


**FIGURE 7. Snf1 regulation of Gcn4 abundance requires Gcn2 and Gcn20.** A and B, cells expressing HA-tagged Gcn4 protein, in combination with either wild-type (+) or complete deletions ( $\Delta$ ) of the *SNF1*, *GCN2*, or *GCN20* genes as shown, were grown in SC-Ura medium containing 2% glucose. Protein extracts were prepared and analyzed by Western blotting with antibodies against either the HA epitope or Sse1.

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. 7, A and B, lane 2). However, when *GCN2* or *GCN20* was deleted, Gcn4 levels were no longer induced in the absence of Snf1 (Fig. 7, A and B, lane 3). Deletion of *SNF1*, *GCN2*, or *GCN20* did not induce any HA-reactive bands in the absence of Snf1 (data not shown). Therefore, induction of Gcn4 in *snf1* $\Delta$  strains requires Gcn2 and Gcn20, indicating that Snf1 control of Gcn4 levels occurs at a step prior to activation of eIF2 $\alpha$  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



**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-Gcn20 complex and attenuated by phosphorylation of Gcn2 at Ser-577 (6, 45). The TOR kinase pathway inhibits Gcn2 by promoting phosphorylation of Ser-577. Dephosphorylation of Ser-577 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 Ser-577 (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-Gcn20 complex.

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 40-min 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 *snf1* $\Delta$  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 an *snf1Δ* strain found that over 400 genes require Snf1 for high expression when cells are grown for 6 h in rich media containing low glucose levels (0.05%). Although 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.* (3) study 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 12-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 an *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  $\beta$ -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 6 h. 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*<sup>+</sup> and *snf1Δ* cells for invertase activity (11), Mig1 phosphorylation (11), inositol auxotrophy (40), or pseudohyphal growth (41). Collectively, our results and the results of 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 eIF2 $\alpha$  (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 Ser-577 (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 Ser-577 (43). Under conditions that inactivate the TOR pathway, such as rapamycin treatment, a Tap42-PP2A or Tap42-Sit4 phosphatase complex can dephosphorylate Ser-577 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 Ser-577 (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 dependence of Gcn4 translational induction on Gcn2 suggested that the levels of phosphorylated eIF2 $\alpha$  should be elevated in *snf1Δ* strains. Unexpectedly, our initial assays have revealed similar eIF2 $\alpha$ -P levels in *SNF1* and *snf1Δ* strains grown in SC media (data not shown). This finding may indicate that Snf1 impacts Gcn2 and/or eIF2 $\alpha$  activity by both positive and negative mechanisms, as has been proposed for Sit4 (6), negating a detectable effect on eIF2 $\alpha$ -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 eluci-

## Snf1 Regulation of Gcn4

date the cross-talk in pathways that regulate glucose repression and amino acid biosynthesis.

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