INTEGRATION OF GENERAL AMINO ACID CONTROL AND TOR REGULATORY PATHWAYS IN YEAST

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DEDICATION

I would like to dedicate this thesis to the memory of my kid sister Erin who passed away in July of 2000. The strength and courage she exhibited during her long childhood illness was an inspiration to me and others.

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ABSTRACT

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Two important nutrient sensing and regulatory pathways, the general amino acid control (GAAC) and the target of rapamycin (TOR), participate in the control of yeast growth and metabolism in response to changes in nutrient availability. Starvation for amino acids activates the GAAC through Gcn2p phosphorylation of the translation initiation factor eIF2 and preferential translation of *GCN4*, a transcription activator. TOR senses nitrogen availability and regulates transcription factors, such as Gln3p. We used microarray analyses to address the integration of the GAAC and TOR pathways in directing the yeast transcriptome during amino acid starvation and rapamycin treatment. We found that the GAAC is a major effector of the TOR pathway, with Gcn4p and Gln3p each inducing a similar number of genes during rapamycin treatment. While Gcn4p activates a common core of 57 genes, the GAAC directs significant variations in the transcriptome during different stresses. In addition to inducing amino acid biosynthetic genes, Gcn4p activates genes required for assimilation of secondary nitrogen sources, such as γ-amino-butyric acid (GABA). Gcn2p activation upon shifting to secondary nitrogen sources is suggested to occur by means of a dual mechanism. First, Gcn2p is induced by the release of TOR repression through a mechanism involving Sit4p protein phosphatase. Second, this eIF2 kinase is activated by select uncharged tRNAs, which were shown to accumulate during the shift to GABA medium. This study highlights the

mechanisms by which the GAAC and TOR pathways are integrated to recognize changing nitrogen availability and direct the transcriptome for optimal growth adaptation.

Ronald C. Wek, Ph.D., Chair

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ABBREVIATIONS

3-AT	3-amino-triazole
bZIP	basic zipper
c-terminus	carboxy terminus
DSE	downstream sequence element
DTT	dithiothreitol
eIF	eukaryotic initiation factor
ER	endoplasmic reticulum
GAC	GCN4p activation core
GAAC	general amino acid control
GCN	general control nonderepressible
GCRE	general control responsive element
GRC	GCN4p repression core
HAT	histone acetyltransferase
HisRS	histidyl-tRNA synthetase
HRI	hemin-controlled repressor
α-IPM	α-isopropylmalate
ISR	integrated stress response
LacZ	β-galactosidase gene
Met-tRNA$_i^{Met}$	initiator methionyl-tRNA
Min	minute(s)
MSX	L-methionine sufoximine
mRNA	messenger RNA

mTOR	mammalian target-of-rapamycin
NaF	sodium fluoride
NMD	nonsense mediated decay
Rap	rapamycin
PCR	polymerase chain reaction
PEK	pancreatic eIF2 kinase
PERK	PKR-like ER kinase
PIKK	phosphatidylinositol kinase-related kinase
PKR	double-stranded RNA-activated kinase
PMSF	phenylmethylsulfonyl fluoride
qRT	quantitative reverse transcription
RM-A	Reveromycin A
RT	reverse transcriptase
Rp	ribosomal protein
SC	synthetic complete
SD	synthetic dextrose
S.E.	standard error
SM	sulfometuron methyl
STE	stabilizer element
TC	ternary complex
TF	transcription factor
TOR	target-of-rapamycin
TORC1	TOR complex 1

TORC2	TOR complex 2
TSC	Tuberous sclerosis complex
TAF	TBP-associated factor
TBP	TATA-binding protein
UAS	upstream activation sequence
uORF	upstream open reading frame
UPR	unfolded protein response
UTR	untranslated region

INTRODUCTION

I. The eIF2 kinase family

Eukaryotic cells regulate protein synthesis in response to diverse environmental cues by down-regulating overall translation or in some cases increasing translation of specific mRNAs. This response is regulated in large part by a family of protein kinases that phosphorylate the eukaryotic initiation factor 2 (eIF2) on serine-51 of the alpha subunit (1). Four mammalian eIF2 kinases have been identified, including the Heme regulated inhibitor (HRI), Double-stranded RNA activated protein kinase (PKR), Pancreatic eIF2 kinase (PEK) or PKR-like ER kinase (PERK), and the general control nonderepressible (GCN2) protein kinase (Fig. 1). This latter kinase is expressed broadly among eukaryotes, including the yeast *Saccharomyces cerevisiae*, and will be discussed in much greater detail below. This family of eIF2 kinases display extensive sequence homology in their catalytic kinase domains, but contain divergent regulatory regions outside this domain allowing for stress-specific activation of kinase function (Fig. 1).

The eIF2 protein consists of three subunits (α, β, and γ) and forms a ternary complex (TC) with GTP and initiator methionyl-$tRNA_i^{Met}$ facilitating binding of initiator tRNA to the 40S ribosomal subunit (2) and ribosomal selection of the translational start site (3-4). Coupling of the 60S ribosomal subunit to form an 80S initiation complex at the AUG start codon proceeds with hydrolysis of GTP and release of eIF2-GDP (3). In yeast and mammalian mRNAs, upstream open reading frames (uORFs) present in the 5'-UTR of mRNAs allow for gene-specific translational control, a mechanism which will be discussed in further detail in below. The eIF2-GDP is subsequently recycled back to the GTP bound form by the guanine nucleotide exchange factor eIF2B. Phosphorylation of

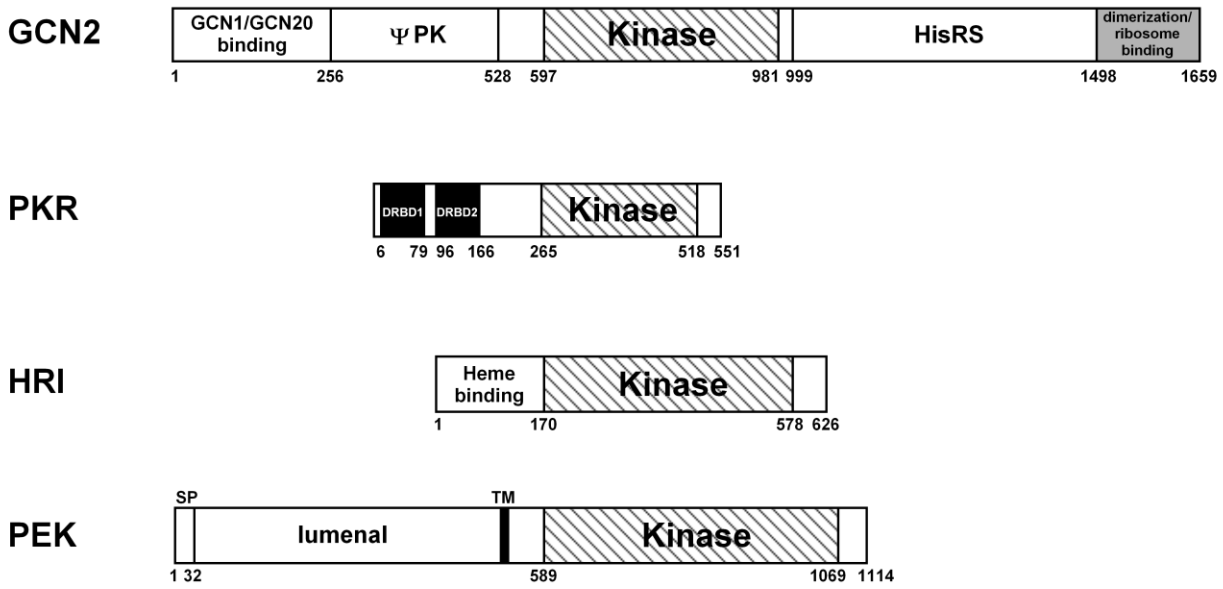


Figure 1. The eIF2 protein kinase family. Schematic diagram of yeast GCN2, human PKR, rat HRI, and mouse PEK or PERK. Conserved catalytic kinase domains are depicted by cross-hatched boxes. Divergent regulatory domains which allow for stress specific activation of these kinases are juxtaposed to the protein kinase domain. As illustrated in the figure, Gcn2p regulatory sequences include the amino terminal region that binds the positive regulators Gcn1p/Gcn20p, pseudo kinase domain, protein kinase region, HisRS-related region and the c-terminal dimerization and ribosome binding sequences. PKR and HRI have dsRNA-binding motifs and heme-binding regions, respectively. PERK/PEK has a transmembrane domain (TM) and a signal peptide (SP) sequence, which flank the ER lumenal region. Participation of the flanking regions in the regulation of each eIF2 kinase is described further in the text.

eIF2 converts eIF2-GDP from a substrate to an inhibitor of eIF2B, resulting in a reduction in TC levels and reduced protein synthesis (3,5).

As mentioned above, the individual eIF2 kinases contain unique regulatory motifs allowing for activation of these kinases resulting in eIF2α phosphorylation in a stress-specific fashion. For example, reduced heme levels in erythroid cells results in activation of HRI, allowing for coordinated translational synthesis of globin in accordance with heme availability (6-7). Unfolded proteins in the endoplasmic reticulum activate PEK or PERK, considered a major effector of the unfolded protein response (UPR) pathway (8-10). Double-stranded RNAs present in virally infected cells result in activation of PKR (11-12) while starvation for amino acids, glucose, serum, or UV irradiation among other stresses results in activation of GCN2 protein kinase (13) (Fig. 2). Inactivation of the TOR signaling pathway in yeast by the immunosuppressant drug rapamycin also results in activation of GCN2 protein kinase (14-16), and the biological significance of this regulatory pathway is a major focus of this thesis.

II. The general control pathway in yeast

Changes in nutrient availability direct programs of gene expression, which allow for adaptive modifications in metabolism and nutrient uptake. Many different stress response pathways are thought to recognize nutritional deficiencies and contribute coordinately to the restructuring of the transcriptome. An important example of such a stress response is the general amino acid control (GAAC) pathway. In the GAAC, starvation for amino acids triggers phosphorylation of eIF2α by the protein kinase Gcn2p (17-19). Ultimately this results in increased expression of a large number of genes

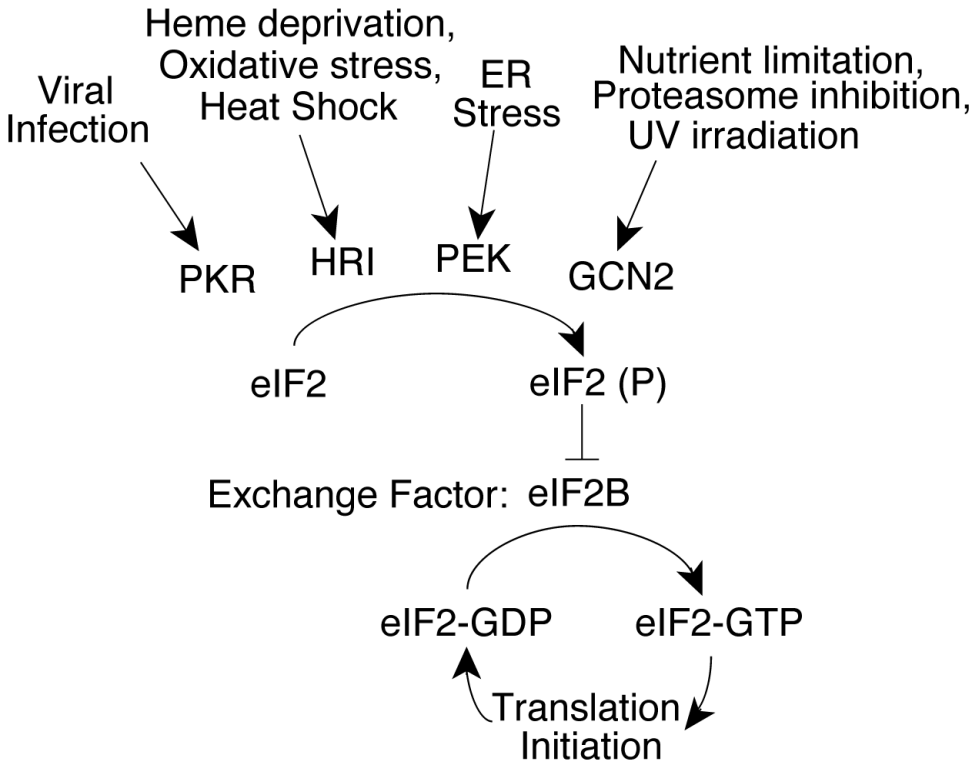


Figure 2. The eIF2 kinases regulate translation in response to diverse environmental stresses. Phosphorylation of eIF2α on Ser-51 by the various eIF2 kinases in response to environmental stresses converts eIF2-GDP from a substrate for the guanine nucleotide exchange factor eIF2B to a competitive inhibitor resulting in lowered ternary complex levels and reduced translation initiation

involved predominantly in metabolism of amino acids. This has been referred to as cross-pathway control since the induction of genes important for the biosynthesis of virtually all amino acids is independent of which amino acid is limiting.

The GAAC can be divided into three basic parts. The first concerns the mechanism by which cells monitor amino acid levels. This sensing mechanism is carried out by the protein kinase Gcn2p and involves direct interaction between Gcn2p and uncharged tRNA that accumulates in cells severely limiting for amino acids (19). The second part involves elevated levels of the transcriptional activator Gcn4p in response to starvation for amino acids. A central feature of this induced expression involves preferential translation of *GCN4* mRNA, a mechanism that has become a classic example of gene-specific translational control. The third part of the GAAC is the coordinate expression of hundreds of genes through Gcn4p-directed regulation of transcription (20). To mediate this regulation of mRNA synthesis, Gcn4p binds to a defined promoter sequence (TGABTVW), referred to as the general control response element (GCRE) and enhances access for the RNA polymerase II transcriptional apparatus. This results in the activation of a collection of genes important for stress remedy and the salvaging of nutrients important for renewal. In addition to amino acid limitation, activation of the GAAC pathway occurs during other environmental stress conditions including other nutrient limitations such as carbohydrate or purine deprivation (13). The mechanistic details central to each of these parts of the GAAC will be described in detail below. Importantly, many of these conceptual features are conserved not only in Gcn2p-mediated stress pathways among other eukaryotic organisms, but also more generally in

other stress management pathways whereby complex stress conditions are recognized and processed to coordinate gene expression.

III. Uncharged tRNA activates Gcn2p protein kinase

Starvation for any one of at least ten different amino acids studied induces expression of Gcn4p and its target genes. Mutations in aminoacyl-tRNA synthetase genes, such as *HTS1* important for charging of of $tRNA^{His}$, elicit the general control response in yeast even in the presence of abundant cognate amino acid (21). Hence, elevated levels of uncharged tRNA that accumulate during amino acid starvation are thought to be the direct signal that activates the general control pathway (22).

The sensor for uncharged tRNA levels in yeast is the multi-domain protein Gcn2p (Figs. 1 and 3). The central kinase domain of Gcn2p is directly involved in catalyzing the phosphorylation of eukaryotic initiation factor-2 (eIF2) in response to stress, an event that as described further below modifies the activity of this translation initiation factor and triggers increased Gcn4p synthesis (23). Recognition and activation of Gcn2p by elevated levels of uncharged tRNA involves a regulatory domain that has sequence homology with almost the entire length of the histidyl-tRNA synthetase (HisRS) enzymes (24). Genetic studies support the idea that the HisRS-related domain of Gcn2p participates in the monitoring of these starvation conditions as residue substitutions in the HisRS domain were shown to effect *GCN4* expression. In particular one mutant gcn2-m2 contains residue substitutions in motif 2 (Y1119L and R1120L), a conserved region among class II synthetases that directly interacts with tRNA substrates (21). The *gcn2-m2* mutant was not able to phosphorylate eIF2α and failed to induce expression of *GCN4*

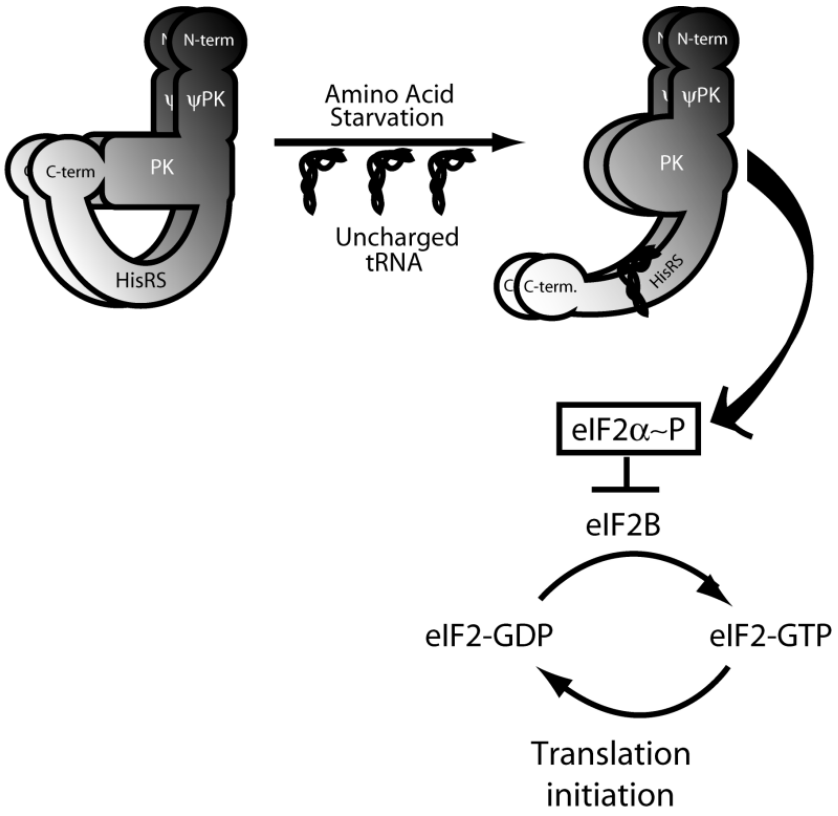


Figure 3. Uncharged tRNA activates Gcn2p protein kinase. Uncharged tRNA binds to the HisRS domain in Gcn2p protein kinase resulting in activation of kinase activity. This stimulates phosphorylation of the eIF2α at Serine-51 converting eIF2 to a potent inhibitor of the guanine nucleotide exchange factor eIF2B. This results in reduced translation initiation and leaky scanning of ribosomes on mRNAs.

or its target genes in yeast cells starving for any one of at least six different amino acids (21,25). In addition, motif 2 alterations also significantly reduced binding of uncharged tRNA to the HisRS-related domain of GCN2 *in vitro* (21,26). The extreme c-terminus of Gcn2p is multi-functional, and it has been suggested that its ability to dimerize is central to facilitate the HisRS-domain binding to uncharged tRNA (26). Gcn2p binding with uncharged tRNA is not restricted to uncharged $tRNA^{His}$; therefore sufficient divergence from the bona-fide HisRS enzyme has occurred to allow for binding of many different uncharged tRNA species that accumulate during amino acid starvation conditions. Furthermore, Gcn2p has reduced affinity for aminoacylated tRNA *in vitro*, consistent with the idea that it is activated by only uncharged tRNA (26).

Induction of Gcn2p by uncharged tRNA is proposed to involve a transition from an inhibited to catalytically active conformation that is signaled by direct contacts between the protein kinase domain, HisRS-regulatory region, and the extreme c-terminus of Gcn2p (27-28). Biochemical and genetic studies examining the dynamic interactions between the domains of Gcn2p suggest that there is inhibitory contact between the protein kinase domain and the Gcn2p c-terminus that is relieved upon binding of uncharged tRNA to the HisRS-related domain (26-27,29). However, release of this inhibitory interaction does not appear to be sufficient for induced eIF2 kinase activity. Association of uncharged tRNA with Gcn2p is also thought to contribute to a positive-acting contact between the amino terminal portion of the HisRS-region and the protein kinase domain (27,29) (Fig. 3). Interaction between the HisRS and protein kinase regions is proposed to realign kinase subdomains V and VIb, including residues Arg794 and Phe842, opening the substrate binding cleft of the catalytic domain and allowing for eIF2

binding and phosphorylation. Located amino-terminal to the Gcn2p catalytic domain is a second region sharing homology with protein kinases (Fig. 1). This so-called partial or pseudo-kinase domain is required for induction of eIF2α phosphorylation in response to amino acid limitation. Supporting the model that release of the autoinhibitory interaction between the protein kinase and extreme c-terminal regions of Gcn2p is not sufficient for activation is the apparent lack of eIF2 kinase activity of the Gcn2p kinase domain by itself. However, this isolated Gcn2p kinase domain becomes hyperactive after residue substitutions in the key subdomains V and VIb of Gcn2p (R794G and F842L, designated Gcn2p-Hyper) that are proposed to direct an active conformation independent of interaction with the HisRS-related or partial kinase regions (29). More recent evidence suggests that a network of hydrophobic interactions centered on Leu-856 results in autoinhibition by constraining the critical alpha C helix in the kinase domain which is subsequently released by tRNA binding and autophosphorylation of Thr-882 in the activation loop (30).

Accompanying this activated conformation of Gcn2p is autophosphorylation at threonine residues 882 and 887 in the so-called activation loop (T-loop) in subdomain VII of the kinase domain (31). This autophosphorylation may occur in *trans* between Gcn2p dimers. Dimerization of Gcn2p appears to occur independent of amino acid starvation, and involves predominantly the extreme c-terminus of Gcn2p, as well as weaker contributions between the HisRS-related and protein kinase domains (Fig. 3). Upon self phosphorylation, Gcn2p is presumed to retain its induced eIF2 kinase activity until it is dephosphorylated by protein phosphatases. Dephosphorylation of eIF2α is thought to be mediated by a type I protein phosphatase encoded by *GLC7* (32). The

activity of the Glc7p is regulated by multiple regulatory proteins that associate with this phosphatase, enhancing its recognition for phosphorylated protein substrates. In mammalian cells, this process is carried out by the regulatory protein Gadd34, which itself is induced in response to eIF2α phosphorylation, as part of a feedback mechanism controlling stress gene expression (33-35). It remains to be determined whether a Gadd34 orthologue functions in the regulation of eIF2α phosphorylation and *GCN4* expression in fungi.

There have also been reported examples of stress induction of *GCN4* expression independent of Gcn2p. Induction of Ras2p which leads to activation of protein kinase A in yeast is suggested to increase Gcn4p synthesis (36). Furthermore, defects in tRNA processing or nuclear transport enhance *GCN4* translation independent of eIF2 phosphorylation (37-38). As will be described in the results section, a substantial induction of *GCN4-lacZ* reporter gene activity is observed in *gcn2*Δ cells grown in media containing an alternative nitrogen source. The mechanistic details of this Gcn2p-independent induction of *GCN4* expression is not known, but it may involve direct or indirect reduction in eIF2 activity. However, this thesis will show that the GAAC is not only essential to regulating the transcriptome in response to nutrient deprivation, but also for directing gene expression by shifting to alternative nitrogen sources in the growth medium.

IV. Ribosome association contributes to Gcn2p protein kinase function

Targeting of Gcn2p to the ribosomal machinery contributes to the mechanism by which Gcn2p monitors the levels of uncharged tRNA in cells. Association with

ribosomes occurs through the extreme c-terminus of Gcn2p (39-40). A second interface between ribosomes and Gcn2p involves the N-terminal of Gcn2p from residues 1 to 272 (41-43). This region interacts with a protein complex consisting of Gcn1p and Gcn20p, a complex which is also is associated with ribosomes (41,44).

Several models have been proposed to explain how ribosomal binding can contribute to activation of Gcn2p protein kinase in response to starvation for amino acids. First, ribosome targeting may facilitate Gcn2p access to its substrate eIF2. Arguing against this idea is the observation that the hyper-activated kinase domain of Gcn2p itself efficiently phosphorylates eIF2 *in vivo* despite the absence of ribosome association (29). Furthermore, as discussed further below, the gcn2p-605 mutant which fails to associate with ribosomes induces eIF2α phosphorylation and *GCN4* translation in response to glucose deprivation (45). Consequently, ribosome association of Gcn2p is not absolutely obligatory for eIF2 access *in vivo*.

A second model has been proposed that describes a role for ribosome targeting by Gcn2p activity involves its requirement for dimerization and trans-phosphorylation. Ribosome targeting could elevate localized concentrations of the eIF2 kinase polypeptide, thus enhancing the proximity of the Gcn2p that would accentuate the formation of dimers. Dimer formation would then facilitate trans-autophosphorylation at the activation loop of the eIF2 kinase domain. Such a model has been proposed for the related eIF2 kinase PKR that participates in an anti-viral defense pathway in mammalian cells (46). Opposing this model is the observation that the dimerization of Gcn2p through it c-terminus is quite stable independent of amino acid availability (15).

Therefore, a role for a dynamic equilibrium between Gcn2p monomers and dimers in the mechanism of eIF2 kinase regulation appears unlikely.

A third model for ribosome targeting of Gcn2p that emphasizes the interaction between this eIF2 kinase and the Gcn1p-Gcn20p complex revolves around the idea that levels of uncharged tRNA are best measured in the context of the ribosome itself. Gcn1p is proposed to be localized in proximity to the A site of ribosomes, and the role of Gcn1p as a positive regulator of the GAAC may reside in its ability to eject uncharged tRNA that enters the ribosome during elongation (47). Such evicted uncharged tRNA would be transferred by the Gcn1p-Gcn20p complex to the HisRS-related domain of Gcn2p, eliciting the active conformation of this eIF2 kinase. While uncharged tRNAs have been shown to bind in a codon-dependent manner to the A site of eukaryotic ribosomes, the levels of uncharged tRNA required to facilitate such binding *in vivo* have not yet been resolved (48). The Gcn1p-Gcn20p complex may serve to increase the binding of uncharged tRNA to ribosomes. The amount of Gcn1p is much lower than ribosomes in yeast, and therefore only a portion of total ribosomes are associated with Gcn1p. If Gcn1p is overexpressed in yeast, which would facilitate the proposed binding of uncharged tRNA to ribosomes, there is enhanced sensitivity to the aminoglycoside antibiotic paromomycin, a drug that reduces translation fidelity (47).

Strongly supporting the model that the Gcn1p-Gcn20p complex is critical for optimal activation of Gcn2p eIF2 kinase activity in response to amino acid starvation is the observation that deletion of *GCN1* blocks eIF2 phosphorylation by Gcn2p and the resulting induction of translation of *GCN4* mRNA (49). As observed for *gcn2* mutants, including those removing the c-terminal domain of Gcn2p, deletion of either *GCN1* or

GCN20 render cells hypersensitive to growth inhibition in response to amino acid deprivation (41,49-50). Additionally, this proposed regulatory linkage between Gcn2p and Gcn1p appears to be conserved throughout evolution, as orthologues for both proteins are found in a range of organisms, including fungi, *Caenorabditis elegans*, *Drosophila melanogaster*, *Arabidopsis*, and mammals. By contrast, orthologues for the transcription activator Gcn4p are restricted to certain fungi, although related basic-zipper (bZIP) transcriptional regulators may carry out an analogous function in *S. pombe* and higher eukaryotes. Finally, studies have identified a protein designated Yih1p (IMPACT in mammals) that appears to compete with Gcn2p for the Gcn1p positive regulator (51-52). Although the precise biological scheme regulating Yih1p is still not understood, these studies suggest that Gcn2p binding with Gcn1p/Gcn20p may be an important mechanism regulating the GAAC in response to selected stress arrangements.

V. Phosphorylation of eIF2 induces *GCN4* translational control

Translational control of *GCN4* mRNA is the major mechanism directing expression of this transcriptional activator in response to nutrient limitation. *GCN4* translation is enhanced by phosphorylation of eIF2α by Gcn2p. As noted above, ternary complexes consisting of eIF2 bound to GTP and initiator Met-tRNA$_i^{Met}$ participate in ribosomal selection of the start codon. During this translation initiation process, GTP associated with eIF2 is hydrolyzed to GDP and eIF2 is released from the ribosome. Recycling of eIF2-GDP to the GTP-bound active form requires a guanine nucleotide exchange factor, eIF2B (Fig. 3). Gcn2p phosphorylation of eIF2α at Ser51 converts this initiation factor from a substrate to an inhibitor of the eIF2B, reducing the levels of eIF2-

GTP available for translation initiation (17,53-54). The guanine nucleotide exchange factor eIF2B consists of five polypeptide subunits designated eIF2B α-ε that are organized into catalytic and regulatory sub-complexes (55). Guanine nucleotide exchange is catalyzed by Gcd6p (ε) with the assistance of Gcd1p (γ). Phosphorylated eIF2 associates tightly to the regulatory sub-complex consisting of Gcn3p (α), Gcd7p (β) and Gcd2p (δ), preventing eIF2 association with the catalytic sub-complex and blocking GDP-GTP exchange (53,55-56).

Control of *GCN4* translation initiation is mediated by four uORFs located in the 5'- non-coding portion of the *GCN4* mRNA (Fig. 4). These uORFs, numbered from 1 through 4, are each only two or three codons in length. Studies involving analysis of different configurations of the 5'-leader of the *GCN4* mRNA fused to a *lacZ* reporter gene in yeast, and *in vitro* measurements of ribosome association at different locations along the leader of the *GCN4* mRNA, support the following model (17,57). Translation of the *GCN4* mRNA begins in a cap-dependent fashion with the scanning ribosome initiating at the 5'-proximal uORF1. Upstream ORF1 serves as a positive-acting element in *GCN4* translational control by allowing ribosomes. to reinitiate at downstream ORFs. The basis for the reinitiation capacity is thought to reside in the termination context; sequences 3' to the uORF1 stop codon are proposed to facilitate the retention of the small ribosomal subunit with the *GCN4* mRNA (58). Following translation of uORF1, 80S ribosomes are proposed to decouple while retaining association with the 40S subunit with the termination region of the leader of the *GCN4* mRNA. The small ribosomal subunit resumes scanning in a 5' to 3' direction along the leader of the *GCN4* mRNA. When eIF2-GTP is plentiful during the nonstarved state, the small ribosomal subunit quickly

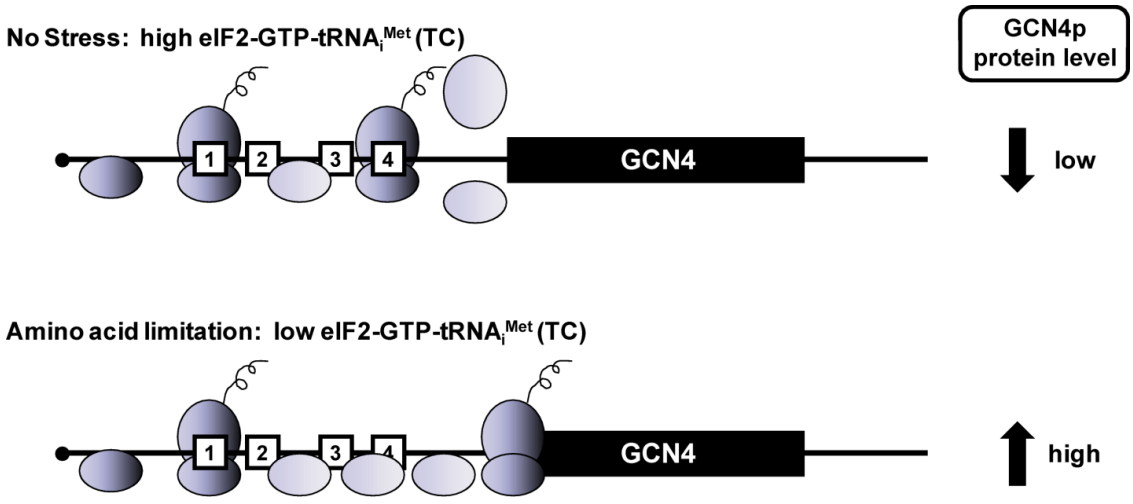


Figure 4. Gene-specific translational control of *GCN4* mRNA. Schematic depicting the translational regulation of *GCN4* mRNA. The *GCN4* mRNA is illustrated by the line, with the coding region in the black box and the four uORFs numbered 1 – 4 (open boxes) indicated in the 5'-leader of the transcript. In non-stressed (*upper*) cells when TC levels are high, the 40S ribosomal subunit (depicted in light grey) is retained following translation of uORF1 by 80S ribosomes (dark grey) and reinitiates at downstream uORFs 2 – 4, thus preventing translation of the *GCN4* coding region and resulting in reduced levels of Gcn4p. In stressed cells (*lower*), reduced levels of TCs due to inhibition of eIF2B activity by phosphorylated eIF2α allow retained 40S subunits to bypass inhibitory uORFs 2 – 4 and allow reinitiation at the *GCN4* protein coding region. This results in increased *GCN4* translation and elevated levels of GCN4p transcription factor.

reacquires the eIF2 ternary complex and, coupled with the 60S ribosome, reinitiates translation at uORF2, uORF3 or uORF4. Following translation of one of these three upstream ORFs, the ribosome dissociates from the *GCN4* mRNA, thus blocking expression of the downstream *GCN4* coding region.

During amino acid starvation, when eIF2-GTP levels are reduced, there is a delay in reinitiation following translation of uORF1. The increased time required for reacquisition of eIF2-GTP coupled with Met-$tRNA_i^{Met}$ allows the 40S ribosomal subunit to scan through the negative-acting uORFs 2, 3 and 4. While scanning in the mRNA leader from ORF4 to the initiation codon of the *GCN4* coding region, the ribosome acquires the eIF2 ternary complex, facilitating translational expression of *GCN4*.

With limitation for a single amino acid, such as that observed following the addition of 3-aminotriazole (3-AT), an inhibitor of histidine biosynthesis to yeast cultures, there is enhanced eIF2α phosphorylation and Gcn4p synthesis accompanied by a reduction in both general translation and yeast growth. However, as judged by polysome profiles in sucrose gradient sedimentation experiments, there is not necessarily a significant accumulation of free ribosomal subunits. This suggests that there is not a major block in translation initiation due to the levels of Gcn2p phosphorylation of eIF2α induced by the 3-AT inhibitor (39). This observation indicates that reduced general translation accompanying amino acid limitation can be simply a function of the lowered levels of free amino acids, rather than lowered availability of eIF2-GTP required to sustain general translation initiation. Therefore, stimulation of *GCN4* translation can occur in response to a modest reduction in eIF2-GTP that does impede general translation initiation. Re-initiation of translation that occurs in the *GCN4* mRNA leader may be

particularly sensitive to lowered levels of eIF2 ternary complex accompanying such amino acid limitations.

Reduced translation initiation can occur when a yeast strain auxotrophic for amino acids is shifted from media containing complete amino acids to that deprived of all amino acids (59). Under this severe starvation condition where the strain cannot synthesize its full complement of amino acids there appears to be enhanced activation of Gcn2p and hyperphosphorylation of eIF2α that would further reduce eIF2-GTP levels required to sustain general translation initiation. Constitutively active mutants of GCN2 or expression of high levels of mammalian eIF2 kinases PKR or PERK/PEK in yeast also lead to hyperphosphorylation of eIF2α and a general reduction in protein synthesis (60-63). In these conditions of reduced general translation initiation by Gcn2p hyperphosphorylation of eIF2α there is still enhanced translation of *GCN4* mRNA, although there would be lowered levels of general translation including the synthesis of proteins encoded by genes transcriptionally induced by Gcn4p. Together, these studies indicate that a range of eIF2α phosphorylation levels can induce gene-specific translation, and this translational control can occur in the absence of a general protein synthesis defect. Only after falling below a certain threshold level of eIF2-GTP is there a reduction in general translation.

Regulation of *GCN4* translation by eIF2α phosphorylation is also central to general control pathways in other fungi such as *Candida albicans* and *Neurospora crassa* (64-66). However in these other fungi, the leader of the mRNAs encoding these Gcn4p orthologues have only two upstream ORFs. The first upstream ORF functions similarly to the positive-acting ORF1 in yeast *GCN4* mRNA, while the second upstream ORF is

the sole inhibitory element, preventing ribosomal reinitiation at the downstream coding region during the fed state. This two upstream ORF configuration has been constructed artificially in yeast *GCN4* mRNA by deleting ORFs 2 and 3 (17). The *GCN4* mRNA leader that retains only upstream ORF1 and ORF4 mediates translational control in response to eIF2 phosphorylation, albeit at reduced levels compared to the wild-type version of *GCN4* mRNA containing the full complement of inhibitory upstream ORFs. Translation of the related mammalian bZIP transcription factors ATF4 and ATF5 is also regulated by a mechanism involving delayed translation reinitiation and two upstream ORFs which is analogous to *GCN4* arrangement (67-68).

VI. Multiple regulatory mechanisms control Gcn4p levels in response to starvation for amino acids

While translational control is a major mechanism enhancing Gcn4p levels in response to nutrient depletion, regulation of the synthesis and stability of *GCN4* mRNA and protein turnover also contribute to the overall increase in Gcn4p concentrations. Elevated synthesis of *GCN4* mRNA in response to amino acid limitation in *S. cerevisiae* is modest, with less than a two-fold increase in *GCN4* transcription. As discussed more fully below, other stress conditions can induce *GCN4* translation and glucose limitation or exposure to the drug rapamycin can coincidently enhance as much as a 2 to 3-fold increase in *GCN4* transcription. Furthermore, increased synthesis of mRNA encoding Gcn4p orthologues in other fungi, such as *Candida albicans*, *Neurospora crassa* and *Aspergillus nidulans*, is a significant contributor to overall expression of this transcriptional activator (64-65,69). For example the *GCN4* orthologue in *A. nidulans*,

designated *cpcA*, has an eight-fold increase in its mRNA levels in response to eight hours of exposure to 10 mM 3-AT, compared to only a five-fold increase in the CpcA protein levels (69). An important contributor to this increase in *cpcA* mRNA is autoregulation, whereby CpcA binds to its own gene promoter leading to a further amplification of expression. These studies suggest that enhanced mRNA levels can serve in conjunction with elevated translation to regulate the expression of Gcn4p-related transcription factors.

The fact that the leader of the *GCN4* mRNA contains short ORFs that precede the *GCN4* coding region presents challenges to the stability of this mRNA. Transcripts that contain nonsense mutations within the protein coding region are degraded in yeast by the nonsense mediated decay (NMD) pathway, preventing the synthesis of truncated proteins (70). The NMD pathway degrades not only nonsense-containing mRNAs, but also those with frameshift mutations, improperly-spliced transcripts, and mRNAs containing ORFs preceding a coding region. It is proposed that ribosomes pause at nonsense codons, promoting the assembly of a surveillance complex that upon translation termination scans towards the 3'-end of the transcript. An improper translation termination event is recognized if the surveillance complex detects a specific downstream sequence element (DSE) and associated proteins, leading to assembly of additional factors, including Hrp1p, that facilitate decapping of the transcript by Dcp1p. In the case of the *GCN4* transcript, there is the presence of a stabilizer element (STE) 3' of uORF4 that associates with Pub1p and prevents signaling of the decapping pathway (71). This would maintain stability of *GCN4* mRNA independent of the nutritional status of the cell. However, Pub1p binding with RNA does not appear to impede scanning ribosomes, and therefore does not prevent translation reinitiation at the *GCN4* coding region.

Gcn4p resides predominantly in the nucleus where it is highly unstable with a half-life of less than 5 minutes (72). Upon amino acid starvation there is a stabilization of Gcn4p that, in combination with increased expression of *GCN4*, leads to elevated steady state levels of Gcn4p and enhanced transcriptional activation. Degradation of Gcn4p depends on its ubiquitination by the ubiquitin-conjugating enzyme Cdc34p in combination with the SCF^{Cdc4p} complex (73). Such ubiquitination directs Gcn4p to the proteasome where is it is degraded. Ubiquitination of Gcn4p is induced by the cyclin-dependent protein kinase Pho85p that, in conjunction with its regulatory subunit Pcl5p, targets Gcn4p for ubiquitination by specifically phosphorylating Gcn4p at residue Thr165 (74). Central to the regulation of Pho85p phosphorylation of Gcn4p is the availability of Pcl5p. *PCL5* mRNA is induced in response to nutrient limitation by a mechanism involving transcriptional activation by Gcn4p. However, Pcl5p is thought to be labile, and it is suggested that translation of *PCL5* mRNA is low when there is reduced general translation at the onset of an amino acid starvation condition. Reduced levels Pcl5p would lower Pho85p phosphorylation of Gcn4p and insure the availability of this transcription factor at the onset of a starvation condition. With elevated levels of Gcn4p and increased expression of its target genes, amino acid levels would be replenished in yeast, contributing to increased synthesis of Pcl5p.

Central to this model is the delayed translation of *PCL5* mRNA relative to expression of Gcn4p and at least a portion of its target gene products. This timing of stressed-induced gene expression has not yet been well addressed experimentally. A second cyclin-dependent protein kinase Srb10p is also linked to Gcn4p turnover, and deletion of *SRB10* and *PHO85* together is required for maximum stability of Gcn4p (75).

Srb10p may phosphorylate five distinct residues in Gcn4p, including Thr165. Similar to that described for Pho85p, such Srb10p phosphorylation is thought to mediate degradation of Gcn4p through Cdc34p and possibly the SCF^{Cdc4p} complex. However, regulation of Gcn4p levels by Srb10p appears to be controlled independent of the availability of amino acids.

VII. Gcn4p interacts with the core transcriptional machinery to coordinate gene expression

Gcn4p is a member of the bZIP family of transcription factors. The bZIP region located at the extreme c-terminus of Gcn4p is important for Gcn4p dimerization and binding to GCREs embedded in the promoter regions of target genes (76). While other members of this family such as mammalian ATF4 can heterodimerize with other bZIP proteins, Gcn4p is thought to function primarily as a homodimer (77). Such DNA binding can occur in the absence of nutrient limitation, contributing to the basal expression of Gcn4p regulated genes. This is best illustrated by the observation that while yeast cells deleted for *GCN4* are viable they can no longer grow without all amino acids supplemented in the growth medium. With the increased levels of Gcn4p that are observed during amino acid starvation, there is enhanced Gcn4p binding to GCREs and stimulation of transcription. Early studies on the transcriptional target genes of Gcn4p focused on amino acid biosynthetic genes (76). These have been expanded by microarray studies in cells starved for branched-chain amino acids (78) or histidine (79). The latter study will be described in much more detail below. Activation of transcription involves the amino-terminus of Gcn4p which contains seven clusters of hydrophobic residues

interspersed among acidic residues (80). These hydrophobic segments are essential for recruitment of multi-subunit protein complexes, collectively referred to as coactivators (81-82).

Genetic analysis of the viable mutants generated by the *Saccharomyces* Genome Deletion project indicates that at least seven different coactivator complexes can associate with Gcn4p and impact expression of genes subject to GAAC (82). One of the best characterized examples of Gcn4p-coactivator interaction involves the SAGA complex, which contains the histone acetyltransferase (HAT) subunit, Gcn5p (83). Acetylation of nucleosomal H3 and H2B by Gcn5p leads to remodeling of chromatin that exposes or masks binding sites for TATA-binding protein (TBP) and RNA polymerase II in core promoter regions. Along with Gcn5p, SAGA contains TBP-associated factors (TAFs) that directly contribute to recruitment of general transcription factors. Additional Gcn4p co-activator complexes are SWI/SNF and RSC that hydrolyze ATP to displace nucleosomes and alter the availability of protein binding sites in promoters (82,84).

The precise contribution of these coactivators in Gcn4p-mediated induction of transcription is still not completely understood. Clearly, portions of different coactivator complexes can contribute to activation by Gcn4p at individual target gene promoters. For example, mutations in multiple subunits of seven different coactivators, including SAGA, SWI/SNF, and RSC, lowered the induced levels of *HIS4* and *SNZ1* mRNA in response to amino acid limitation compared to transcription in wild-type cells (82). Surprisingly, among the SAGA subunits characterized, only Gcn5p was dispensable for increased expression of these Gcn4p target genes in response to amino acid starvation. By comparison, significant induction of *ARG1* expression required four coactivators, RSC,

CCR4/NOT, SRB/MED and PAF1 complex (THO/TREX), with SAGA and SWI/SNF being dispensable (82). However, ChIP experiments measuring recruitment of Gcn4p-associated proteins to the chromatin of the *ARG1* promoter region indicated that SAGA and SWI/SNF1, in addition to SRB/MED, were strongly associated in response to amino acid starvation conditions. Less pronounced, albeit significant, Gcn4p-dependent immunoprecipitation in the *ARG1* promoter region was observed for coactivators SRB/MED and PAF1 complex. These results suggest that Gcn4p can recruit more coactivators to a given target promoter than is required for full expression in response to nutrient deprivation (82). Given that Gcn4p activates hundreds of genes, Gcn4p may interact with many different coactivators to overcome diverse regulatory arrangements in target promoters. It is unlikely that these large multi-subunit coactivator complexes reside at a given promoter simultaneously. Perhaps each coactivator binds transiently, contributing their specific functions at the promoter and dissociating prior to entry of a different coactivator complex. Furthermore, the subunit composition of coactivator complexes may vary between different promoter contexts, with certain subunits being dispensable for coactivator complex function or combining differentially to form diverse complex arrangements.

While GCREs are an important feature of Gcn4p-mediated activation of gene transcription, almost half of the genes which were shown to be induced by four-fold or more following treatment with 3-AT and dependent on Gcn4p function had no recognizable binding element in their promoter region or sequences upstream of their translation start site (20,79). It is certainly possible that these genes have GCREs in the transcribed portion of the gene (intron or exon regions) which have functional

significance for transcriptional induction. An alternative explanation is that transcriptional control of these genes by Gcn4p is indirect. As described below, Gcn4p induces the expression of a large collection of transcriptional activators. Furthermore, Gcn4p could modulate transcription through protein-protein interactions that are independent of GCRE binding at a regulated gene. For example, Gcn4p could bind and inactivate transcriptional factors that mediate repression of genes void of GCREs.

While Gcn4p is predominantly viewed as an activator of transcription, DNA microarray analysis of Gcn4p-dependent gene expression in 3-AT treated cells has suggested that Gcn4p can also contribute to repression of transcription (79). For example as studied further in this thesis, there is a dependence on Gcn4p for repressed transcription of genes encoding ribosomal proteins or translation factors in response to amino acid starvation conditions. Since not all of these genes have recognizable GCREs in their promoter regions, it was speculated that Gcn4p probably contributes indirectly to their repressed expression. This is further supported by the observation that overexpression of Gcn4p in the absence of nutrient limitation also reduces transcription of ribosomal protein genes (85). Collectively, expression of these genes are reduced during amino acid starvation conditions by mechanisms involving the transcriptional regulator Rap1p and signal pathways controlled by protein kinase A and Tor proteins (84,86-90). It is proposed that elevated levels of Gcn4p may enhance transcriptional repression in concert with these regulatory pathways by Gcn4p binding and sequestering transcription factors required for expression of ribosomal proteins and translation factors (79). Gcn4p is also reported to enhance expression of protein kinases and phosphatases, providing for a range of possible mechanisms that could modulate these signaling

pathways. Finally, Gcn4p is linked to expression of a large number of other transcription factors that together could combine for direct and indirect Gcn4p control of diverse stress pathways. One such factor, Uga3p, a zinc-finger containing transcription factor required to induce expression of genes involved in the catabolism of GABA as a secondary nitrogen source, is a central focus of experiments in this thesis.

Gcn4p has been termed the "master regulator" of a five layered program of gene regulation designed to alleviate nutrient deprivation (20,79). Certainly, the core layer of Gcn4p transcriptional control involves genes directly contributing to the synthesis of amino acids, a group of target genes that had been widely studied previously (76). As noted above, general control is a true cross-pathway stress response in that starvation for a single amino acid, such as histidine, induces the expression of genes directly involved in the synthesis of all 20 amino acids. It has been confirmed for a large number of these amino acid biosynthetic genes that their encoded enzyme activities are induced as part of the general control program. Natarajan et al. (79) reported that of the 539 genes whose transcription requires Gcn4p for full induction in response to amino acid depletion, only 73 contribute to amino acid biosynthesis. Therefore, the influence of Gcn4p exceeds beyond core amino acid synthetic genes, a key point that will be emphasized in latter sections of this dissertation.

The second layer of gene regulation by Gcn4p involves intermediary metabolism related to amino acid biosynthesis and nutrition (79). For example, it was reported that following 3-AT treatment, 16 genes were induced that function in the synthesis of vitamins that are important cofactors for enzymes in pathways related to amino acids. Expression of several genes encoding amino acid permeases are induced by Gcn4p

following amino acid starvation, including the mRNA for the general amino acid permease Gap1p, a protein critical to nitrogen regulation in yeast. In addition, mRNAs for the basic amino acid permease Can1p, and a broad spectrum permease Agp1p were also induced. Of the 35 members of the mitochondrial carrier family involved in metabolite transport between this organelle and the cytoplasm, 10 are regulated transcriptionally by Gcn4p. Given that portions of the synthetic pathways for arginine, lysine and the branched chain amino acids are carried out in the mitochondria, it is rationalized that the availability of such transport systems is linked to the demands of the amino acid biosynthetic pathways. Another organelle associated with Gcn4p-directed gene expression is the peroxisome. The peroxisome has a primary role in the β-oxidation of fatty acids and detoxification which may be linked with a yeast cell strategy for coping with amino acid depletion. With regards to amino acid biosynthesis, lysine synthetic enzymes Lys1p and Lys4p are located in the peroxisome, and certain peroxisomal mutants in Pex8p and Pex15p are impaired for synthesis of lysine (91). Therefore, Gcn4p-directed expression of peroxisomal-related genes may contribute to enhanced lysine production.

It is of interest to note that Gcn4p also induces five purine biosynthetic genes in response to amino acid limitation, and as further discussed below purine deprivation is a potent inducer of eIF2 phosphorylation and *GCN4* translational control (79,92). The physiological basis for this regulatory linkage may involve the metabolic overlap between the biosynthesis of certain amino acids and purines. For example, the purine ring of ATP is utilized early in the histidine biosynthetic pathway, and induced purine synthesis by Gcn4p may be important for supporting enhanced histidine production. The

connection between histidine and purine synthesis is also highlighted by the regulation and function of *HIS7* encoding glutamine amidotransferase cyclase (93). This bifunctional enzyme catalyzes the fifth and sixth step in the histidine synthetic pathway and produces the by-product 5-aminoimidazole-4-carboxamide ribotide (AICAR) which is an important intermediate in the purine pathway. Regulation of *HIS7* involves Gcn4p binding to two GCREs, designated 1 and 2, in its promoter region that work synergistically to induce transcription. In response to adenine limitation, a second transcriptional activator Bas1p in complex with Bas2p is thought to bind GCRE-2 and activate *HIS7*. Both Gcn4p and the Bas1p/Bas2p complex are required for maximal expression of *HIS7* in response to combined starvation for amino acids and purines. *BAS1*, which functions to activate the transcription of multiple purine biosynthetic genes, is itself transcriptionally regulated by Gcn4p (79). Therefore, Gcn4p contributes to increased expression of *HIS7* both directly and through enhanced expression of other stress-related transcription factors.

The third layer of the Gcn4p-mediated program of gene regulation involves control of the translational machinery. As highlighted above, Gcn4p is required for reduced expression of a number of translation factors and some 90 ribosomal protein genes (79). Such a reduction in the synthesis of the translational machinery would be appropriate with the reduced cellular growth rates associated with lowered amino acid availability. This regulatory strategy is analogous to the well described stringent response in *Escherichia coli* that represses synthesis of rRNA and subsequently ribosomal proteins in response to amino acid starvation (94). It is interesting to note that in both *E. coli* and yeast, the signal for this repression is proposed to be placement of

uncharged tRNA into the A sites of ribosomes. By contrast, Gcn4p enhances the expression of a number of different aminoacyl-tRNA synthetases genes following 3-AT treatment, including *KRS1*, *ILS1* and *MES1*, suggesting a mechanism to enhance the efficiency of aminoacylation of their corresponding cognate tRNAs during conditions of reduced amino acids (79). Interestingly, as described earlier, RM-A, an inhibitor of isoleucyl-tRNA synthetase induces *ILS1* mRNA diminishing growth inhibition by this compound (95). However, aminoacyl-tRNA synthetases are not uniformly induced by histidine limitation. In fact, genes encoding GlnRS, PheRS, and SerRS are repressed in cells exposed to 3-AT. Therefore, the role of Gcn4p and its impact on aminoacylation of different tRNA species has yet to be resolved.

A fourth layer of Gcn4p-mediated control involves broader themes in cellular stress responses. Starvation for various nutrients, including limiting nitrogen or carbohydrates, induces a process of autophagy that facilitates the bulk turnover of cytoplasmic material. Autophagosomes, which have many parallels to lysosomes in higher eukaryotes, deliver cytoplasmic proteins and organelles to yeast vacuoles, where they are degraded and reclaimed for later use (96). Gcn4p induces the expression of two vacuolar proteases and three autophagy proteins, including the protein kinase Apg1 and its associated protein Apg13 that are instrumental for activating the autophagy process (79). The absolute requirement of *GCN4* for autophagy remains controversial. In response to histidine starvation, autophagy was reported to occur independent of Gcn4p function (79). By contrast, Talloczy et al. (97) suggested that autophagy in response to nitrogen starvation is blocked in yeast strains devoid of *GCN4* or *GCN2*. The apparent conflict between the two studies may lie in the different yeast strains utilized or in the

different nutrient stresses used to invoke autophagy. It has been observed that nitrogen limitation is a potent inducer of autophagy, while starvation for an amino acids such as tryptophan leads to accumulation of fewer autophagic bodies (98). A further complication is that while nitrogen starvation induces eIF2 phosphorylation by Gcn2p, reportedly there is no synthesis of Gcn4p (99). The mechanistic basis for the absence of Gcn4p expression during nitrogen deprivation is not understood, but it is reasoned that complete starvation for nitrogen would thwart amino acid biosynthesis.

General nutrient starvation also leads to accumulation of glycogen. This polymer of glucose begins to accumulate as nutrients begin to be depleted, allowing yeast cells to accumulate carbohydrates to be utilized upon resumption of vegetative growth or during spore germination (100). Gcn4p contributes to the expression of several proteins involved in glycogen accumulation, including glycogen synthase, glycogenin and the branching enzyme (79). Glycogen synthesis and turnover varies considerably during the time course of a nutrient-depletion study. Utilizing matched yeast cultures shifted from synthetic medium containing 2% to 0.05% glucose, a condition that induces eIF2 phosphorylation by Gcn2p and *GCN4* translation, it was found that there was a 7-fold increase in glycogen levels after two hours of culture incubation (45). Accumulation of glycogen was similar between strains containing wild-type or deleted *GCN2* function. However, following 22 hours of incubation in the glucose-deficient media, glycogen levels were more significantly reduced in the absence of Gcn2p activity, with the *gcn2* mutant cells having four-fold less glycogen than the wild-type strain. Therefore, the general control has important roles in both amino acid and carbohydrate metabolism.

The fifth and final layer of Gcn4p-directed gene expression involves the induction of signaling proteins such as protein kinases, protein phosphatase catalytic and regulatory subunits, and transcription factors. Activation of these regulatory genes may allow for amplification of the general control response, and provide for a means of communication with other stress response pathways. In the case of protein kinases, it was noted above that Gcn4p induces expression of Apg1 protein kinase which is important for eliciting autophagy (79). Autophagy is also positively regulated by Snf1p protein kinase, required for glucose derepression, and negatively impacted by nutrient sensing protein kinases Tor and Pho85p (96). Therefore, multiple nutrient stress response pathways interconnect to control this catabolic trafficking process. Another protein kinase whose expression is induced by Gcn4p is Npr1p. Npr1p promotes the stabilization Gap1p, a general amino acid permease, and the proteolysis of the tryptophan permease Tat2p (89). As described above Gcn4p activates *GAP1* expression, and the added *NPR1* expression may further contribute to the cellular uptake of amino acids. Npr1p is also inhibited by Tor-directed protein phosphorylation, further emphasizing cross pathway control of key nutrient regulatory steps. In the example of phosphatase-related genes, Gcn4p directs expression of Gip1p, a Type 1 protein phosphatase interacting protein required for synthesis of spore walls (79). Sporulation in yeast occurs in response to starvation for nitrogen in the absence of a fermentable carbon source, and the linkage between Gcn4p and spore wall formation may suggest that Gcn4p can contribute to a range of stress response options in yeast.

Gcn4p enhances expression of 26 different transcription factors involves in a broad range of stress response. The largest collection of transcription factors are

involved in amino acid and purine biosynthesis, including Arg80p, Bas1p, Gln3p, Leu3p, Lys14p, Met4p, and Met28p (79). This suggests that the general control pathway can be fine tuned to accommodate transient cellular requirements for individual amino acids. As noted above Bas1p is critical for expression of purine biosynthetic genes and the histidine pathway gene *HIS7*. The example of Leu3p nicely illustrates this idea of superimposition of pathway specific regulation onto general control. Leu3p binds to promoter elements in a large number of genes involved in branched chain amino acid synthesis and represses transcription (101). In the presence of the leucine biosynthetic precursor α-isopropylmalate (α-IPM), Leu3p becomes an activator of transcription. Levels of α-IPM are subject to the availability of leucine through feedback inhibition of α-IPM synthase, the *LEU4* product. Therefore, leucine levels would dictate whether Leu3p functions as an activator or repressor. In response to 3-AT treatment, Gcn4p also induces expression of genes encoding transcription factors involved in peroxisome proliferation (*PIP2*, and see discussion in the third layer of Gcn4p-direction gene expression), utilization of poor nitrogen sources (*GAT1* and *UGA3*), heat shock (*HSF1*), maltose catabolism (*MAL13*), and meiosis (*RIM101*) (79). Utilization of alternative nitrogen sources is a major theme of this thesis. Overall, this collection of transcription factors may work in concert with Gcn4p, insuring that the timing and content of stress gene expression is appropriately tailored to a mosaic of stress inputs.

The multi-layered model of target gene induction described above was developed in large part through the use of large scale microarray studies in cells treated with 3-AT to elicit starvation for histidine (79). We propose that the Gcn4p-directed transcriptome may be differentially modulated during different nutrient-related stress conditions. In this

way, the resulting gene expression could elicit metabolic patterns that best alleviate the specific underlying stress. To address this idea, in this thesis we compared GAAC-directed transcription changes during 3AT treatment with inactivation of TORC1 with rapamycin or growth in the presence of alternative nitrogen sources.

VIII. The general control pathway and yeast physiological strategies

Yeast can synthesize each of its twenty amino acids *de novo*. However, in rich medium replete with amino acids, yeast import amino acids and reduce the levels of Gcn4p-directed gene expression. This insures that yeast do not synthesize biosynthetic enzymes unnecessarily, and facilitates a rapid doubling time. When there is an imbalance of amino acids in the medium, yeast will enhance biosynthetic genes to generate the required metabolites. In the laboratory, addition of 3-AT or sulfometuron methyl (SM), a chemical inhibitor of branched chain amino acid biosynthesis, induces high levels of Gcn4p and its target genes (21). The yeast GAAC induces expression of not only genes required for the biosynthesis of the limiting amino acids but also those involved in synthesis of non-limiting amino acids. Much of this analysis has measured transcript levels and many of these genes have not yet been assessed for induced protein synthesis. Furthermore, given feedback inhibition for many of these biosynthetic pathways, expression of these many genes does not necessarily dictate that there is increased flux through each pathway. Nevertheless, it appears that yeast has coupled a central mechanism for induction of many biosynthetic pathways to a single signal- accumulation of uncharged tRNA. Zaborske et al. (22) has used a genome-wide analysis of tRNA charging to determine that starvation for individual amino acids can lead to elevated

levels of deacylated tRNAs representing both the limiting and non-limiting amino acids. This suggests that the cross-regulation also extends to the proposed uncharged tRNA activating signal.

Previous studies from this lab suggest that an important goal of the GAAC pathway is storage of nitrogen in the form of amino acids in response to severe nutrient deprivation (45). Upon glucose deprivation, yeast enhance their overall levels of free amino acids, with an elevation in the vacuolar amino acid pool and a concomitant depletion in the cytoplasmic amino acid levels. Concentrations of individual amino acids in the vacuole vary, with glutamate constituting nearly a third of the total pool, and arginine and alanine each constituting about 10% (102). Initially this increase in vacuolar amino acid levels is independent of Gcn2p activity (45). But with longer periods of glucose limitation, the levels of vacuolar amino acids are much reduced in *GCN2*-deficient cells compared to wild-type. As noted above, such glucose limitation increases eIF2 phosphorylation and *GCN4* translational, and these results suggest that induced general control contributes to the storage of amino acids when carbohydrates are limiting and there is reduced protein synthesis and cell growth. The storage of amino acids during nutrient limitation would provide yeast cells ready access to nitrogen when the carbohydrates again become accessible (45). Importantly, vacuolar storage of amino acids is also triggered in response to reduced assimilation of ammonia, indicating that this storage strategy is invoked in response to diverse nutrient stress conditions (102).

Accumulation of glycogen also occurs in yeast when glucose or amino acids begin to be depleted in the culture media and yeast cells have reduced their rate of growth (100). As highlighted above, the maintenance of accumulated glycogen levels is

dependent on Gcn2p protein kinase activity, suggesting that general control has a broad role in storage of nutrients (45). It is noted that loss of *GCN4* is suggested to enhance glycogen accumulation as judged by iodine staining of cells grown on synthetic dextrose agar medium (79). This may suggest some differences between *GCN2* and *GCN4*-deficient strains. However, the timing in such iodine staining experiments is critical as glycogen synthesis and turnover change during growth phases. *GCN4*-deficient strains have a clear requirement for amino acid supplements for robust growth, and the absence of this transcription factor could enhance nutrient starvation signals that trigger early activation of glycogen synthase. This in turn would lead to earlier glycogen accumulation in *gcn*4 mutant strains compared to wild-type cells. Similarly, vacuolar amino acid levels are enhanced in *gcn4* mutant cells grown to mid-logarithmic phase in synthetic medium in the absence of amino acid supplements as compared to wild-type strains (45). With the addition of all twenty amino acids to the medium, there is a reduction in the vacuolar amino acid pool in the *gcn4* mutant, albeit the amino acid levels still remain significantly higher than that measured in non-starved wild-type cells. Therefore, the loss of *GCN4* function is itself a stress that can trigger certain coping strategies. By comparison, *GCN2*-deficient cells, which retain some basal expression of Gcn4p, are not amino acid auxotrophs and can provide an important alternative tool to assess the contribution of the induced general control response to stress conditions.

Gcn4p orthologues are also required for longer term strategies for dealing with nutritional stress in other fungi. For example in the dimorphic fungi *Candida albicans*, Gcn4p facilitates filamentous growth in response to amino acid starvation. Such filaments enable this fungi to forage for new resources when nutrients are depleted (64).

In *Aspergillus nidulans*, the related Gcn4p transcription factor CpcAp functions in a cross pathway control, and serves to block formation of cleistothecia or fruit bodies when nutrients are limiting (103). Formation of these complex reproductive structures consumes macromolecules and energy, and induced CpcAp expression would signal that inadequate nutrients are present to carry out this process.

IX. Multiple stresses activate Gcn2p eIF2 kinase activity

Phosphorylation of eIF2 by Gcn2p occurs in response to diverse nutrient limitations, including starvation for different amino acids, purines, or glucose. Accumulation of uncharged tRNA is thought to be the activating signal for each of these starvation condition because yeast expressing *gcn2-m2*, defective for binding to uncharged tRNA, are unable to induce eIF2α phosphorylation and mediate *GCN4* translation control. Purine starvation may elicit elevated uncharged tRNA levels by reducing ATP levels or the biosynthesis of certain amino acids, such as histidine, or by altering the processing of tRNA. In the case of glucose limitation, accompanying energy reductions or the lowering of amino acid levels in the cytoplasm that accompany vacuole accumulation of amino acids could lead increased uncharged tRNA. While 60S ribosomal association is thought to be obligate for activation of wild-type Gcn2p during amino acid limitation, it appears to be largely dispensable in response to glucose limitation (45). Furthermore, the requirement for Gcn20p is not essential for induced *GCN4* expression during this deprivation for this carbohydrate. These results suggest that uncharged tRNA is an important signaling molecule that activates Gcn2p in response to many different nutritional limitations. However, there may be differences between the

mechanisms by which uncharged tRNA is delivered to and recognized by Gcn2p during amino acid and carbohydrate deficiency.

In addition to nutrient limitation, many other stress conditions have been recently reported to activate Gcn2p eIF2 kinase activity. Exposure of yeast cells to high concentrations of sodium, volatile anesthetics, the immunosuppressant rapamycin, methyl methanesulfonate (MMS), or tunicamycin have been reported to induce eIF2 phosphorylation, and many of these conditions have also been shown to elevate *GCN4* translation (14,16,79,104-105). Two fundamental questions come to mind concerning the induction of eIF2 phosphorylation by these diverse stress conditions. First, what are the mechanisms activating Gcn2p in response to these diverse stresses? And second what is the physiological rationale for inducing Gcn4p in response to this range of cellular stress conditions. We still do not yet have clear answers to these questions, although there are several clues that provide insight into likely explanations. These clues and insights are provided below.

The first question concerns the signals that activate Gcn2p in response to this diverse collection of stress conditions. In the examples of sodium toxicity, anesthetics and rapamycin there is a linkage to amino acid metabolism. Exposure to anesthetics and elevated concentrations of sodium are suggested to impair uptake of amino acids by yeast (104-105). Uptake of leucine or tryptophan is inhibited by anesthetics, and the enhanced levels of these amino acids to the medium can overcome the growth defect associated with anesthetics. Furthermore, yeast cells prototrophic for amino acid biosynthesis are resistant to anesthesia. Together these observations support the idea that the physiological action of anesthesia involves amino acid uptake in cells and nutrition

availability. Along similar lines, the immunosuppressant Tacrolimus (FK506) functions independently of calcineurin to block tryptophan uptake resulting in Gcn2p activation (106). An inihibitor of isoleucyl-tRNA synthetase, Reveromycin A (RM-A), activates Gcn2p kinase activity and *GCN4* translation (95). The example of activation of Gcn2p by high levels of sodium is complex. While elevated concentrations of either sodium or potassium reduce yeast uptake of phenylalanine and leucine, only sodium induces eIF2 phosphorylation and *GCN4* translational control (104). Furthermore, general control in yeast strains prototrophic for amino acids is still activated by the addition of high sodium concentrations to the medium (15). These results suggest that sodium stress is induced by a mechanism other than nutritional starvation due to reduced uptake of amino acids. The identity of one or more of these alternative sodium stress signals is currently not known. However, it was recently reported that elevated levels of sodium enhanced the levels of uncharged tRNAs for selected amino acids, supporting the idea uncharged tRNA binding is at least one reason for activation of Gcn2p during this stress conditions (22). A curious note regarding sodium stress is that deletion of *GCN2*, or other general control genes such as *GCN1,* confer growth resistance to sodium (104). Such sensitivity may indicate that sodium induces hyperphosphorylation of eIF2 and a block in translation. By deleting the eIF2 kinase, protein synthesis would not be impeded by sodium stress. By contrast, deletion of *GCN4* is reported to confer growth sensitivity to sodium (104). Gcn4p activates transcription of *HAL1*, an important regulator of salt balance, through antagonism of Sko1p repressor. The apparent phenotypic difference whereby *gcn2* mutant cells are growth resistant, and *gcn4* mutant cells are sensitive has also been reported for rapamycin treatment (14).

As will be discussed further below, Tor has a central role in linking protein synthesis and cell growth and division to nutrient sufficiency, and rapamycin complexes with the immunophilin-related protein Fpr1 to inhibit this protein kinase in yeast (89,107). Interestingly, rapamycin stimulates eIF2 phosphorylation by Gcn2p in non-starved cells by blocking Tor-mediated phosphorylation of Gcn2p at serine-577, located upstream of the kinase domain (14). Tor phosphorylation of Gcn2p is not thought to be direct, but rather through an unknown protein kinase that is downstream of Tor and Tap42p-regulated type 2A and type-2A-related protein phosphatatases. Phosphorylation of Gcn2p at serine-577 reduces its binding to uncharged tRNA, while an alanine substitution at serine-577 contributes to induced Gcn2p eIF2 kinase activity independent of nutrient availability. Induction of Gcn4p increases expression of amino acid biosynthetic genes, and contributes in concert with Tor to activation of genes required for catabolism of poor nitrogen sources, as well as repression of genes encoding ribosomal proteins and translation factors. Thus, rapamycin-mediates dephosphorylation and activation of Gcn2p, and this linkage between the Tor and Gcn2p provides a mechanism of cross-talk between different nutrient sensing pathways, the latter point being addressed by experiments in this dissertation. It is noteworthy that the nutritional stresses characterized, amino acid or purine starvation, do not contribute to dephosphorylation of Gcn2p at serine-577 (14); therefore, the precise nutritional stress modulating Tor-directed control of general control remain to be determined. Uncharged tRNA may be a contributing signal to activation of Gcn2p by rapamycin, given that yeast containing *gcn2-m2* are blocked for induction of *GCN4* translation in response to rapamycin

exposure. However, there is currently no evidence to support the idea that rapamycin treatment alters the efficiency of aminoacylation of tRNA.

The final examples of stress agents which activate Gcn2p that will be discussed are MMS and tunicamycin. MMS induces DNA damage, and DNA microarray analysis indicates that this alkylating agent induces expression of genes involved in synthesis and repair of DNA and detoxification, and represses those functioning in the synthesis of nucleotides, RNA and ribosomes (108). Interestingly, over 90 genes involved in amino acid biosynthesis are induced by twofold or more, suggesting a linkage between MMS and the general control pathway. Indeed, MMS enhances eIF2α phosphorylation by Gcn2p and *GCN4* translation (79). This induction mechanism is blocked in yeast containing the *gcn2-m2* mutation or defects in *GCN1* or *GCN20*, supporting the model that uncharged tRNA is a contributing signal to activation of Gcn2p in response to MMS. Furthermore, mutations in checkpoint proteins that are required for response to DNA damage, e.g. Rap53p, do not reduce MMS induction of *GCN4* expression (79). This suggests that important signaling pathway required for repair of DNA damage are not involved in activation of Gcn2p.

Proteins are substrates for MMS alkylation which can lead to impaired activity of enzymes, such as aminoacyl-tRNA synthetases. However to our knowledge, there have not been any reports of increased uncharged tRNA levels in yeast treated with MMS. It is also noted that oxidized proteins are ubiquitinated and degraded in proteasomes, and impaired proteasome function in mammalian cells through drug treatment or by overexpression of proteins containing poly-glutamine sequences can lead to ER stress linked to the eIF2 kinase PERK/PEK (109). One mechanism by which the ER secretory

pathway manages misfolded protein in the ER lumen is to evict such proteins back to the cytoplasm for ubiquitin-mediated degradation. Proteasome dysfunction in the cytoplasm would block such protein degradation, contributing to a backup of misfolded protein in the ER lumen that elicits an ER stress response. Perhaps, MMS-damaged proteins in yeast also overload the proteasome, contributing to not only a cytoplasmic stress but also stress in the ER. In the case of the ER transmembrane protein PERK/PEK, the ER chaperone GRP78 binds to the lumenal portion of PERK/PEK and represses its cytoplasmic eIF2 kinase activity (110-111). Misfolded protein in the lumen of the ER is proposed to titrate GRP78 from PERK/PEK, facilitating oligomerization and trans phosphorylation that induces eIF2 kinase activity. The cytoplasmic chaperone Hsp90p binds yeast Gcn2p and is proposed to have a critical role for Gcn2p maturation and regulation (112). Following the model proposed for PERK/PEK and ER stress, perhaps Hsp90p inhibition of Gcn2p is relieved by accumulation of damaged protein that selectively titrates the cytoplasmic chaperone from mature Gcn2p. Removal of Hsp90 from Gcn2p may contribute to an activated conformation and elevated eIF2 kinase activity. Altered proteasome function has also been proposed to alter efficient turnover of proteins that could modify Gcn4p activity or expression (113).

Tunicamycin blocks protein glycosylation in the ER and contributes to misfolded protein in this organelle (114-115). Unlike mammals, *S. cerevisae* has only a single eIF2 kinase Gcn2p. However, yeast does have Ire1p, a transmembrane ER protein kinase that shares sequence similarities with mammalian PERK/PEK in its ER lumenal regions (116). Ire1p activates the expression of Hac1p, a transcriptional activator of genes important for ER protein folding and secretion. In mammals, PERK/PEK

phosphorylation of eIF2 serves to reduce general translation, preventing further synthesis of secretory proteins that would further overload the ER. Additionally, PERK/PEK induces expression of the transcriptional activator *Atf4* and its target genes in mammalian cells subjected to ER stress (67). Given that PERK/PEK is not present in yeast it was assumed that this portion of the ER stress pathway was absent from fungi. However with the observation that tunicamycin can activate Gcn2p eIF2 kinase activity, it is inviting to speculate that Gcn2p has assumed a translational regulation role during ER stress (79). Glucose deprivation in mammals also induces ER stress and PERK/PEK activity (114-115). Therefore, the observation discussed above that glucose deprivation can induce eIF2 phosphorylation in yeast may further support this ER stress linkage with Gcn2p. It is curious that a DNA microarray study measuring tunicamycin-induced gene expression in yeast did not identify Gcn4p-directed amino acid biosynthetic genes (117). This may indicate that during ER stress, Gcn2p functions predominantly to regulate general translation, and there is a decoupling of *GCN4* expression. Genetic variations between yeast strains could also impact the different contributions of the general control on ER stress responses.

X. Integration of the general control pathway and TOR signaling in nitrogen assimilation in yeast

As noted above, another stress response pathway that is important for monitoring nutrient availability involves the target-of-rapamycin (TOR) signaling pathway (118). In its natural environment, yeasts are subjected to wide fluctuations in nutrients and the TOR signaling pathway is a central mechanism by which cellular metabolism and growth

is coordinated with environmental cues (119). TOR is a large serine/threonine protein kinase belonging to the phosphatidylinositol kinase-related kinase (PIKK) family (120) and as a critical regulator of cell growth that regulates multiple cellular processes such as transcription, translation, and remediation pathways such as autophagy (89). Yeast contains two homologous TOR protein kinases, Tor1p and Tor2p, which form two distinct complexes, designated TOR complex 1 (TORC1) and TOR complex 2 (TORC2), both of which have parallel functions in mammalian cells (119). TORC1 consists of Kog1 (Raptor), Lst8, Tco89, and either TOR1 or TOR2 protein kinase. TORC2 is composed of only TOR2 and Lst8, Avo1, Avo2, Avo3 (Rictor), Bit2, and Bit61. Many of the proteins present in yeast TORCs have direct homologues in higher eukaryotes (121). The macrocyclic lactone rapamycin, when bound to its FKBP12 receptor (Fpr1p in yeast) inhibits the nutrient sensitive TORC1 (Fig. 5), but not TORC2 (122-124). In an interesting parallel between yeast and mammals, TORC1 phosphorylates Sch9p to regulate ribosomal protein gene expression, analogous to activation of S6K1 in mammals (125). While TORC2 has been shown to activate Ypk2p, an AGC family kinase required for actin polarization (126), similar to activation of PKB/Akt (127).

Nitrogen metabolism is a particularly important pathway regulating TOR function in yeast (128-129). Yeasts are capable of using some 30 different compounds as a nitrogen source including ammonia and all twenty amino acids (130). Thus, all nitrogen-containing constituents of the cell can be derived from degradation of a carbon source and these compounds (131). Among these many compounds, glutamine is the preferred nitrogen source and represents a key intermediate in nitrogen metabolism (131). The

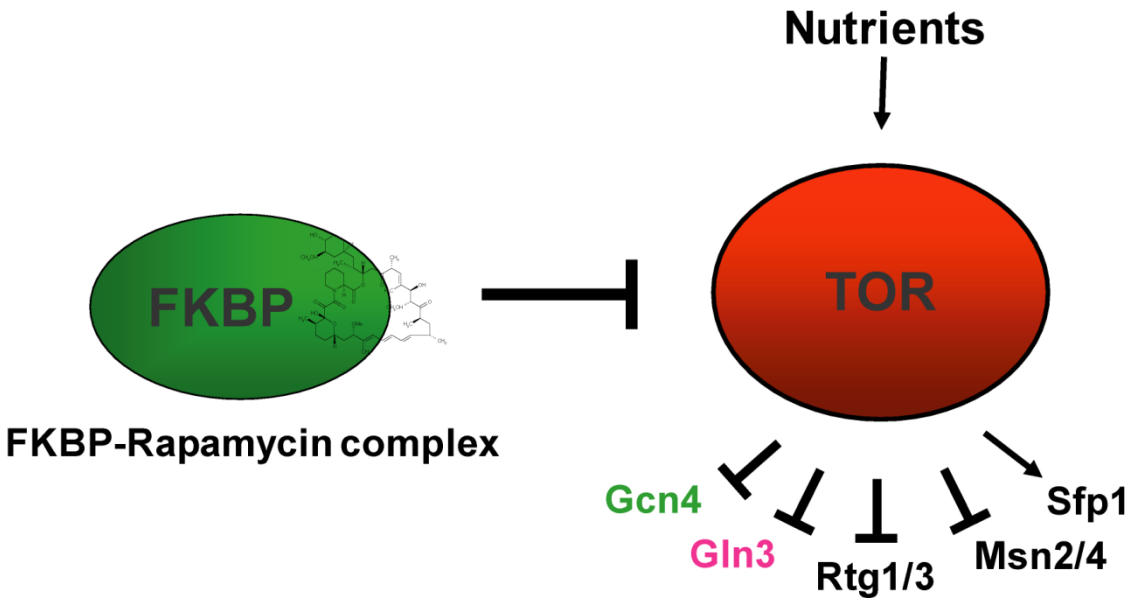


Figure 5. TOR is a key regulator of nutrient-sensitive transcription factors. The immunosuppressant drug rapamycin binds to FKBP (Fpr1p in yeast) which in turn inhibits TORC1. TOR regulates the activity of transcription factors involved in stress responses in yeast including Rtg1/3p (retrograde signaling, TCA cycle), Gln3p (nitrogen discrimination), Gcn4p (general amino acid control), Msn2/4p (carbon limitation), and Sfp1p (ribosomal protein gene expression). Starvation for key nutrients results in relief of TOR repression and activation (or inactivation) of these key factors.

general strategy utilized by yeast following uptake of these nitrogenous molecules is catabolism to yield nitrogen in the form of ammonium, glutamate, or both (130-132). Glutamine is synthesized from glutamate and ammonium by glutamine synthetase, the product of the *GLN1* gene. TORC1 is central for selecting which nitrogen source in the media will be utilized. When the preferred glutamine, or in some strains ammonia, is available, yeast cells will not catabolize poorer nitrogen sources, a phenomenon referred to as nitrogen catabolite repression or nitrogen regulation (131). TORC1 regulates many nutrient-sensitive transcription factors (128) (Fig. 5). TORC1 functions to regulate the nuclear translocation/activity of stress-responsive TFs such as Msn2/4p following carbon limitation and Rtg1/3p known to regulate TCA cycle enzymes in response to mitochondrial defects (133). In addition, TORC1 controls localization of several TFs such as Sfp1p, critical for control of expression of ribosomal protein genes (134). Central to this nitrogen selectivity is TORC1 regulation of the GATA family of transcription activators, such as Gln3p, which direct the expression of genes encoding permeases and catabolic enzymes required for utilization of the secondary nitrogen sources (135). Repression of TORC1 in response to changes in nitrogen quality or treatment with rapamycin leads to Gln3p translocation to the nucleus and activation of Gln3p-target genes (135). Described in more detail below, translational expression of *GCN4* mRNA is controlled by TORC1 regulation of GCN2p protein kinase.

While the mechanisms by which nutrient depletion regulates the TOR pathway are not completely understood, sensing of nutrient stress by the GAAC is known to involve direct monitoring of uncharged tRNAs that accumulate during amino acid depletion (13,18,24). As described in detail above, uncharged tRNA binds to a

regulatory region in Gcn2p homologous to histidyl-tRNA synthetase (HisRS) enzymes, producing a conformational change that activates the eIF2 kinase. In yeast, regulation of Gcn2p is also suggested to involve TORC1. Rapamycin can induces Gcn2p phosphorylation of eIF2 by a mechanism involving release of inhibitory phosphorylation of Gcn2p at serine-577 (14). TORC1 is thought to indirectly facilitate the inhibitory Gcn2p phosphorylation by a mechanism involving an unknown protein kinase and by the type 2A-related protein phosphatase (PPase), Sit4p (14). In the later regulatory scheme, rapamycin would release TORC1 inhibition of Sit4p, allowing for dephosphorylation of Gcn2p. Therefore, at least in the case of rapamycin treatment, there is coordinated regulation between the TOR and GAAC pathways. However, whether or not Gcn4p plays a key role in TOR-mediated gene expression is not known. This latter question is a key focus of this dissertation.

Although a clear connection between TOR and Gcn2p protein kinase in yeast has been established, less is known about the regulation of GCN2 by mammalian TOR. Recent studies from this laboratory have shown that a reduction in 4E-BP and S6K1 phosphorylation (two key downstream targets of mTOR (136)) following leucine starvation is blocked in liver from *Gcn2*$^{-/-}$ mice (137). This suggests that there is a direct or indirect linkage between activation of the GCN2 and mTOR pathways in mammalian cells starved for essential amino acids. Mammalian TOR (mTOR) is regulated by diverse stress conditions by a mechanism involving inhibitory TSC1 and TSC2 proteins (138). Increased translation in *Tsc2*$^{-/-}$ mouse embryo fibroblasts was shown to induce endoplasmic reticulum (ER) stress, activating the eIF2α kinase PERK/PEK, (139). In this case, it is suggested that elevated protein synthesis overwhelms the ER processing

capacity, leading to elevated levels of malfolded protein and activation of PERK and the associated unfolded protein response. Interestingly, recent work in *Drosophila* has shown that RNAi knockdown of dGCN2 suppresses the increased cell size associated with knockdown of *Tsc*2 (140). Thus, while the underlying mechanisms are still not fully understood, these results suggest that eIF2α kinases may also be integrated with the mTOR pathway to control gene expression.

METHODS

I. Construction of yeast strains and culture conditions

The *S. cerevisiae* strains used in this study are listed in Table 1 and all are derived from EG328-1A (141). Strains deleted for *GCN2* or *GCN4* or with a substitution of serine 51 in eIF2α (*SUI2-S51A*) have been described previously (45). All other knock-out strains were constructed by PCR-mediated gene replacement, which removed the entire coding region (142). For *Kan*-marked strains, deletion cassettes were amplified by PCR using genomic DNA from the corresponding deletion mutant in the BY4741 strain background (143) that was purchased from Research Genetics or Open Biosystems (Huntsville, AL). Rapamycin, L-methionine sulfoximine (MSX), and all amino acid supplements were obtained from Sigma (St. Louis, MO) and 3-aminotriazole (3-AT) was purchased from Fluka Chemical (Milwaukee, WI).

To minimize any unintended nutrient imbalances, we utilized isogenic strains which were prototrophic (Table 1). Cells were cultured in synthetic medium (SD) containing 2% dextrose and 0.5% ammonium sulfate (144) and supplemented with all amino acids except histidine (SC). This culture arrangement ensured that cells were saturated with amino acids, allowing for similar growth of strains defective for GAAC and TOR pathways. Because 3-AT inhibits the synthesis of histidine, we deleted this amino acid from the non-stressed and rapamycin-treated cultures as well. Omission of this amino acid did not alter gene expression as judged by RT-PCR analysis of mRNA, such as *HIS4* and *GAP1* (Fig. 6). In experiments which analyzed the role of the GAAC and TOR pathways in nitrogen assimilation, amino acids were omitted, and alternative nitrogen sources (phenylalanine or γ-aminobutyric acid (GABA), each at concentrations

of 10 mM) were substituted for ammonia in the SD medium, as indicated. For some experiments, cells. were grown in YPD medium containing 1% yeast extract, 2% peptone, and 2% glucose, as indicated (144).

TABLE 1. Strains used in this study

Strain	Genotype	Reference
EG328-1A	*MATα ura3-52 leu2 trp1*	(141)
RY139	*MATα ura3-52 gcn2*Δ::*LEU2 trp1*	(45)
RY290-3	*MATα ura3-52 gcn4*Δ::*LEU2 trp1*	(45)
RY287	*MATα ura3-52 gcn4*Δ::*LEU2 trp1 SUI2::S51A*	(45)
WY837	*MATα ura3-52 LEU2 TRP1*	This study
WY838	*MATα ura3-52 gcn2*Δ::*LEU2 TRP1*	This study
WY839	*MATα ura3-52 gcn4*Δ::*LEU2 TRP1*	This study
WY840	*MATα ura3-52 LEU2 gln3*Δ::*TRP1*	This study
WY841	*MATα ura3-52 gcn2*Δ::*LEU2 gln3*Δ::*TRP1*	This study
WY842	*MATα ura3-52 gcn4*Δ::*LEU2 gln3*Δ::*TRP1*	This study
WY798	*MATα URA3 LEU2 TRP1*	This study
WY799	*MATα URA3 gcn2*Δ::*LEU2 TRP1*	This study
WY857	*MATα URA3 gcn4*Δ::*LEU2 TRP1*	This study
WY858	*MATα URA3 LEU2 gln3*Δ::*TRP1*	This study
WY859	*MATα URA3 gcn2*Δ::*LEU2 gln3*Δ::*TRP1*	This study
WY860	*MATα URA3 gcn4*Δ::*LEU2 gln3*Δ::*TRP1*	This study
WY895	*MATα URA3 LEU2 TRP1 aro80*Δ::*kanMX4*	This study
WY962	*MATα ura3-52 LEU2 TRP1 aro80*Δ::*kanMX4*	This study
WY937	*MATα URA3 gcn2*Δ::*LEU2 TRP1 aro80*Δ::*kanMX4*	This study
WY938	*MATα URA3 gcn4*Δ::*LEU2 TRP1 aro80*Δ::*kanMX4*	This study
WY933	*MATα URA3 LEU2 TRP1 uga3*Δ::*kanMX4*	This study
WY939	*MATα URA3 LEU2 TRP1 sit4*Δ::*kanMX4*	This study
WY963	*MATα URA3 LEU2 TRP1 uga1*Δ::*kanMX4*	This study
WY936	*MATα ura3-52 LEU2 TRP1 uga3*Δ::*kanMX4*	This study
WY964	*MATα ura3-52 LEU2 TRP1 uga1*Δ::*kanMX4*	This study

II. Construction of plasmids

Plasmids p180 and p227 have been described previously (145). All other *lacZ* reporter plasmids are derivatives of pRS416-*lacZ* which contains a *lacZ* cassette subcloned into the HindIII/XhoI site of pRS416 (146). The P_{GCRE}-*lacZ* reporter plasmid was derived from plasmid pME1112 (147). Briefly, six copies of the GCRE were amplified by PCR and subcloned upstream of a minimal *CYC1* promoter in the reporter plasmid p416-$CYC1_{TATA}$-*lacZ*. The P_{GATA}-*lacZ* reporter plasmid contains two copies of a consensus GATA (GATAAG) derived from the *GLN1* gene (148). The P_{ARO9}-*lacZ*, P_{UGA3}-*lacZ*, P_{UGA1}-*lacZ* reporter plasmids contain the complete upstream noncoding sequences of the *ARO9* (-608 to -1), *UGA3* (-677 to -1), and *UGA1* (-548 to -1) genes, respectively. These DNA segments were subcloned into the *Xba*I/*Eco*RI site of pRS416-*lacZ*. Deletions of the *UGA3* promoter (-371, -300, -200, and -103) were constructed by PCR. Site-directed mutagenesis of the minimal *UGA3* promoter (-300 to -1) was carried out by PCR with oligonucleotides containing the specific nucleotide changes as indicated (Table 2). Plasmid p722 encodes wild-type *GCN2* and the selectable *URA3* gene (60), and p299 includes the mutant version, *gcn2-m2*, which has Y1199L and R1120L substitutions in the HisRS-related domain of Gcn2p, which block binding to uncharged tRNA (15,21). Plasmid pYB41 encoding a GCN4-lacZ reporter with a selectable TRP1 gene was described previously (26,45). Plasmid p1024 (ADH-GCN4-flag) encodes a constitutive ($GCN4^c$) carboxy-terminal flag-tagged allele of *GCN4* subcloned into the XbaI/XhoI site of p416ADH (149). Plasmid p1025 (GCN4-flag) contains the *GCN4* ORF encoding a carboxy-terminal flag epitope and its upstream regulatory sequences (-1000 to -1) subcloned into the XbaI/XhoI site of pRS416 (146). All oligonucleotides

utilized to construct the above plasmids are described below in Table 2. The nucleotide sequences of all amplified regions were confirmed by nucleotide sequencing. All plasmids used in these studies are listed in Table 3.

TABLE 2. Oligonucleotides used to construct plasmids used in these studies

Oligo	Description	Sequence[a]
kwp404	ARO9 promoter	GCTC**TCTAGA**GGGAAGTCATAGTAATAGAT
kwp405	ARO9 promoter	GCTC**AAGCTT**TGAGTCGATGAGAGAGTGTA
kwp417	UGA3 promoter	GCTC**TCTAGA**GCTTTCTTTTCTAATTCT
kwp418	UGA3 promoter	GCTC**GAATTC**ACCTCACTTTAAAAAACT
kwp429	UGA1 promoter	GCTC**TCTAGA**GAAATGTCAATCATTATTGC
kwp430	UGA1 promoter	GCTC**GAATTC**TGTTCTTAGTTATATTTC
kwp531	UGA3 promoter -371 to -1	GCTC**TCTAGA**AACAATAGGACGAAAAAT
kwp532	UGA3 promoter -300 to -1	GCTC**TCTAGA**TATATTTTTTTTTTGGGC
kwp533	UGA3 promoter -200 to -1	GCTC**TCTAGA**GGAGCCAATCGGATTGAC
kwp549	UGA3 promoter -103 to -1	GCTC**TCTAGA**AAAGAAAAAGAAATTACAAT
kwp550	mutagenesis of GATA site	GAAAAATGTGCAAAAG**AGATCT**GGAGCCAATCGGATTG
kwp551	mutagenesis of GATA site	CAATCCGATTGGCTCC**AGATCT**CTTTTGCACATTTTTC
kwp552	mutagenesis of GCRE site	TAGGGCTACCAGG**GTCCGT**CGACTCAAAGAAAAAGAAAT
kwp553	mutagenesis of GCRE site	ATTTCTTTTTCTTTGA**GTCGAC**GGACCCTGGTAGCCCTA

[a]Sequences in bold indicate restriction sites or nucleotides altered by site-directed mutagenesis.

TABLE 3. Plasmids utilized in these studies

Name	Description
p180	low copy URA3 containing GCN4-uORFs-lacZ reporter
p227	low copy URA3 containing GCN4-ΔuORFs-lacZ reporter
pME1112	integrating plasmid containing $GCRE_6$-lacZ reporter
pRS416	low copy URA3 plasmid
p416-$CYC1_{TATA}$-lacZ	lacZ promoter with minimal CYC1 promoter
p416GCRE-lacZ	low copy URA3 containing GCRE lacZ reporter
p416GATA-lacZ	low copy URA3 containing GATA lacZ reporter
pRS416-lacZ	low copy URA3 containing lacZ reporter gene
p416ARO9-lacZ	low copy URA3 lacZ reporter containing ARO9 promoter
p416UGA3-lacZ	low copy URA3 lacZ reporter containing UGA3 promoter
p416UGA1-lacZ	low copy URA3 lacZ reporter containing UGA1 promoter
p416UGA3-371-lacZ	deletion of UGA3 promoter (-677 to -370) lacZ reporter
p416UGA3-300-lacZ	deletion of UGA3 promoter (-677 to -301) lacZ reporter
p416UGA3-200-lacZ	deletion of UGA3 promoter (-677 to -201) lacZ reporter
p416UGA3-103-lacZ	deletion of UGA3 promoter (-677 to -104) lacZ reporter
p416UGA3-300- m1-lacZ	contains mutation in GATA site at -206 of UGA3 promoter
p416UGA3-300- m2-lacZ	contains mutation in GCRE site at -112 of UGA3 promoter
p416UGA3-300- m3-lacZ	contains mutations in GATA and GCRE sites of UGA3 promoter
p722	low copy URA3 plasmid containing wild-type GCN2 allele
p299	low copy URA3 plasmids containing M2 mutant allele of GCN2
pYB41	low copy TRP1 containing GCN4-uORFs-lacZ reporter
p1024	low copy URA3 plasmid containing ADH-GCN4-flag ($GCN4^c$)
p1025	low copy URA3 plasmid containing GCN4-flag

III. Microarray and sequence analysis

Transcriptome analysis was carried out using RNA prepared from cells treated for 1 hour with 10 mM 3-AT, 200 nM rapamycin, or no stress, in quadruplicate as indicated. The 1 hour incubation time was shown to induce maximal expression of *HIS4* and *GAP1* mRNAs as judged by RT-PCR analysis (Fig. 6). RT-PCR analysis was conducted using Superscript one-step RT-PCR with Platinum Taq polymerase (Invitrogen, Carlsbad, CA).

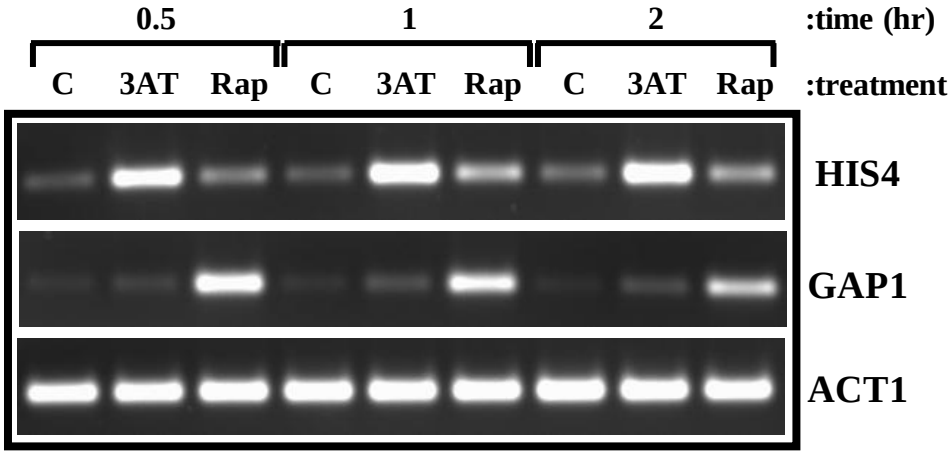


Figure 6. RT-PCR analysis of *HIS4* and *GAP1* transcripts in cells treated with 3-AT or rapamycin. Semi-quantitative RT-PCR measurements of *HIS4*, *GAP1*, or *ACT1* mRNAs were carried out in wild-type (WY798) cells treated with 10 mM 3-AT (3AT), 200 nM rapamycin (Rap), or no treatment (C, control) for 0.5, 1, or 2 hours as indicated. The resulting PCR products were analyzed on a 2% agarose gel and visualized following ethidium bromide staining.

Four independent cultures of strains WY798 (wild-type), WY799 (*gcn2*Δ), WY857 (*gcn4*Δ), WY858 (*gln3*Δ), WY859 (*gcn2*Δ *gln3*Δ) and WY860 (*gcn4*Δ *gln3*Δ) were cultured as described above and total RNA was purified using the hot phenol method (150). The RNA was labeled using the standard Affymetrix protocol for 3'-IVT arrays (Affymetrix, Santa Clara, CA). Labeled cRNA was hybridized for 17 hours to Yeast Genome S98 GeneChips®. Signal values and detection calls were generated using Affymetrix Microarray Suite 5.0. Arrays were scaled to a target intensity of 1000 and detection calls were generated using the default parameters. Average signal intensities from four independent experiments for each of the eighteen treatment groups were compared by performing a Welch's unpaired t-test (151), and false discovery rates (FDR) were calculated according to Benjamini and Hochberg (152). The fold change ratios and *p* values for each probe set and FDR values for each comparison are listed in supplemental Table S1 (153). Microarray data have been deposited in GEO (http://www.ncbi.nlm.nih.gov/geo/) under accession number GSE15254. Pearson and Spearman correlations were calculated using GraphPad Prism version 4.03 (San Diego, CA). The mRNA changes for select genes which were derived from the microarray analyses were independently confirmed by qRT-PCR using SYBR green. Briefly, cDNA was synthesized using 1 µg of total RNA and 2.5 µM random hexamers in the RT mixture according to the manufacturer's protocol (Applied Biosystems, Foster City, CA). Quantitative PCR reactions consisted of 2.5 µl RT product, 400 nM primers, and 1X SYBR® Green PCR master mix (Applied Biosystems, Foster City, CA). Reactions were incubated for one initial cycle at 50 °C for 2 minutes and 95 °C for 10 minutes followed by 40 cycles of 95 °C for 15 seconds and 60 °C for 1 minute. Primers were designed

using PrimerExpress software (Applied Biosystems, Foster City, CA). The nucleotide sequences of the primers used to quantitate the *ARO9*, *ARO10,* and *PDC6* genes, as well as those specific to the *ACT1* gene used for internal normalization, were described by Chen and Fink (154).

Gene list comparisons were performed using the compare classes utility provided by the Regulatory Sequence Analysis Tools (http://rsat.ubl.ac.be/rsat/) (155). Comparisons were made with previous 3-AT and rapamycin data sets (79,134) and to several predefined gene lists, such as genes induced by promoters bound in chromatin immunoprecipitation (ChIP-chip) experiments (156), genes in the MIPS functional catalogue (157), Gene Ontology categories (158), as described by Godard et al. (159). The significance of overlap between gene lists was quantitatively determined by the hypergeometric distribution (160) using the number of probe sets on the S98 array as the population size or by calculating the representation factor (161) using the web utility Microarray Analysis Tools (http://elegans.uky.edu/MA/). Upstream noncoding regulatory sequences were retrieved and analyzed using Regulatory Sequence Analysis Tools (155). The program *dna-pattern* was used to search for and catalogue occurrences of consensus GCRE (TGABTVW) and GATA (GATAAG, GATAAH, GATTA) motifs in yeast promoters. The program *oligo-analysis* (162) was used to search the promoter regions of co-regulated genes for overrepresented sequence motifs. Analysis of the 5'-noncoding regions of the Gcn4p-dependent activation core (GAC) identified the consensus GCRE motif (TGABTVW). Information pertaining to specific gene functions and biological processes was obtained from the Saccharomyces Genome Database (SGD,

http://www.yeastgenome.org) or the MIPS Comprehensive Yeast Genome Database (CYGD, http://mips.gsf.de/genre/proj/yeast).

IV. Immunoblot analysis

Yeast cells were cultured as described above, collected by centrifugation, washed with ice-cold water, and resuspended in a solution of ice-cold 20 mM sodium phosphate [pH 7.2], 50 mM NaCl, 5 mM EDTA, 1 mM DTT, protease inhibitors (100 µM PMSF, 0.15 µM aprotinin, 1 µM leupeptin and 1 µM pepstatin), and phosphatase inhibitors (50 mM NaF and 40 mM β-glycerophosphate). Cells were lysed by vortexing 3-5 times for 30 seconds with glass beads, followed by centrifugation to clarify the lysate. The protein content of the cell lysate was measured using the Bradford method (163). Equal amounts of each protein sample were separated by electrophoresis in a SDS-polyacrylamide gel, and transferred to nitrocellulose filters. Immunoblot analyses were carried out using a polyclonal antibody that specifically recognizes phosphorylated eIF2α at Ser-51 (Research Genetics or StressGen) or M2-FLAG epitope tag (Sigma, St. Louis, MO). Total eIF2α levels were measured using a rabbit polyclonal antibody against recombinant yeast eIF2α (45).

V. LacZ enzyme assays

Yeast cells expressing *lacZ* reporter genes were grown to early logarithmic phase in SD or SC medium and treated as indicated. Following incubation at 30 °C, the non-stressed cells were harvested after 4 hours, and stressed cells were collected after 6 hours, as described previously (45). Cells were collected by centrifugation, resuspended in 250

µl of ice-cold breaking solution (100 mM Tris-HCl [pH 8.0], 20% glycerol, 1mM β-mercaptoethanol and 100 µM PMSF), and broken by vortexing 3-5 times for 30 seconds with glass beads, followed by centrifugation at 15,000 x g to clarify the lysates. To measure the β-galactosidase activity, 5 – 50 µl of extract was added to 950 – 995 µl of Z-buffer (100 mM sodium phosphate [pH 7.5], 10 mM KCl, 2 mM Mg_2SO_4, 4.5 mM β-mercaptoethanol), and the reaction was initiated by the addition of 200 µl solution of o-nitrophenyl-β-D-galactopyranoside (4 mg/ml ONPG in Z-buffer). The reaction was terminated following a 10 – 20 minute incubation at 30 °C by adding 0.5 ml of 1 M Na_2CO_3, and the absorbance of the reaction mixture was measured at A_{420}. Specific enzyme activity is represented as nanomoles of ONPG hydrolyzed per minute per milligram of total protein (nmole/mg/min). Total protein concentration of the clarified lysate was determined using the Bradford method (163). The average β-galactosidase activity ± S.E. from 2 – 3 independent cultures is presented for each experiment. Statistical significance was determined by Student's *t* test.

VI. Polysome analysis

Ribosomal profiles were generated using sucrose gradient centrifugation as described previously (15). Briefly, wild-type or *gcn2*Δ cells were grown in SD medium containing ammonia as the sole nitrogen source and harvested in mid-logarithmic phase. Alternatively, these cells were shifted from SD to GABA medium, cultured for 1 hour, and harvested. Just prior to harvesting, cycloheximide was added to a final concentration of 50 µg/ml to retain polysomes as described (39). Lysate preparation and sucrose gradient analyses were carried out as described previously (39-40). In addition to

cycloheximide, cells lysate preparations contained 10 mM $MgCl_2$, required for association of ribosomal subunits. Aliquots of 20.–.25 A_{260} units were applied to 11.5 ml of 10–50% sucrose gradients and subjected to centrifugation at 40,000 rpm for 2 hours in a Beckman SW41 rotor at 4 °C.

VII. Measurement of tRNA charging

Yeast cells were grown in SD medium and shifted to synthetic medium containing GABA as the sole nitrogen source for 15 minutes or 60 minutes, as indicated. Cells were collected by centrifugation, and tRNA preparations were prepared and analyzed for tRNA charging genome-wide, as described (22). This method involves preparation of RNA from the collected cells using mild acidic conditions, which allow retention of charged tRNAs. The RNA samples were then divided into two equal portions. One part was treated with periodate, which selectively oxidizes uncharged tRNA and selectively blocks their subsequent ligation to a fluorophore-labeled oligonucleotide. The second portion was not treated with periodate, which allows both charged and uncharged portions to be subsequently ligated to the labeled oligonucleotide. Both tRNA preparations were then deacylated using alkaline pH. A fluorescent-tagged oligonucleotide, which contains a stem-loop structure with a portion complementary to the 3'-CCA sequence that is conserved among all tRNAs, was ligated to only those tRNAs with intact 3'-ends. Both samples were labeled with Cy3 or Cy5 fluorophore, and after fluorescent labeling, the tRNA preparations with opposite fluorophores, such as charged tRNA with Cy5 and total tRNA with Cy3, were combined and hybridized to microarrays. The labeled tRNA preparations were hybridized to custom-made microarrays, which contained multiple

replicates for each probe, as described (22). Two microarray analyses were carried out for each sample to minimize dye-bias, the first microarray used Cy5-labeled charged tRNA and Cy3-labeled total tRNA, while the second microarray used Cy3-labeled charged tRNA and Cy5-labeled total tRNA. The array results were presented as histograms of the average relative charging levels ± S.E., with the relative charging level between cells grown in SD medium compared that that cultured in GABA containing medium. The tRNAs were grouped according to amino acid properties (hydrophobic, small, charged, polar). Each tRNA measurement was derived from three independent culture preparations and data analysis and statistics were provided by the scanner software (Axon Instruments, Foster City, CA) and the median values of dye ratios for each array probe are presented. In parallel, Northern analyses were performed using 6.5% polyacrylamide acid denaturing gels to separate charged from uncharged tRNAs, as described (164). As a control, tRNAs were deacylated, and the tRNAs were then transferred to filters and hybridized to radiolabeled probe complementary to $tRNA^{Phe}$ (22).

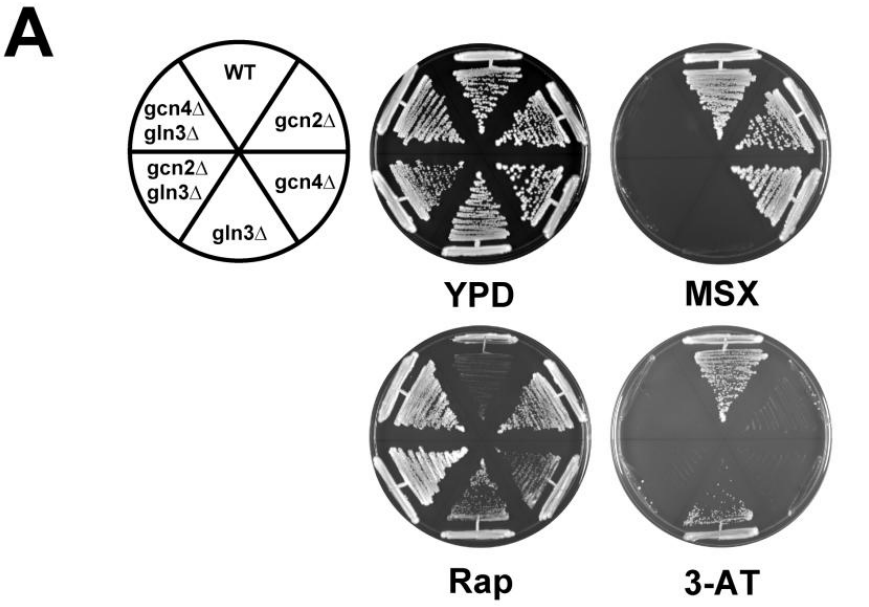
RESULTS

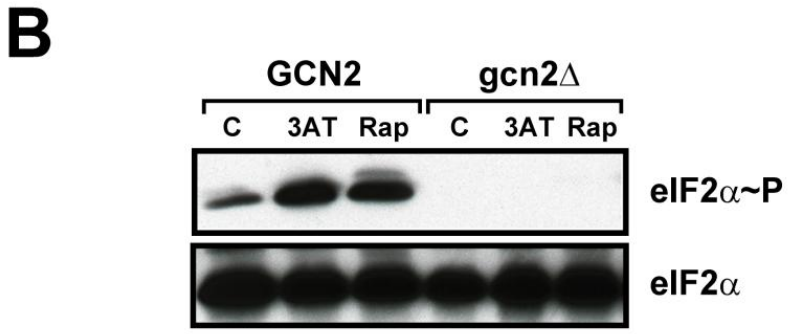
I. Defects in the GAAC and TOR pathways alter growth during nutrient stress

To address the coordination of the GAAC and TOR pathways in the regulation of the transcriptome in response to nutrient stress, we constructed a set of isogenic strains that were deleted for the entire coding region of *GCN2*, *GCN4*, and *GLN3*, individually or in combination (*gcn4*Δ *gln3*Δ and *gcn2*Δ *gln3*Δ). This strain set was derived from strain EG328-1A, which has a robust nutrient stress response, and each strain was prototrophic for amino acid biosynthesis (Table 1).

Deletion of *GLN3* renders cells sensitive to MSX, an inhibitor of glutamine synthetase, which is consistent with the idea that *GLN3* is central for gene expression directed by the TOR pathway in response to nutrient stress (Fig. 7A) (90). By contrast, deletion of *GLN3* renders cells more resistant to rapamycin (Fig. 7A), implying that Gln3p alters genes contributing to control of the cell cycle and proliferation (129).

Consistent with their important roles in the GAAC, deletion of either *GCN2* or *GCN4* resulted in growth sensitivity to 3-AT, a potent inhibitor of histidine biosynthesis (Fig. 7A). Deletion of *GLN3* led to a partial reduction in growth in the presence of 3-AT, suggesting that this transcription activator may function in conjunction with Gcn4p for expression of certain biosynthetic genes. Loss of either *GCN2* or *GCN4* alone resulted in increased resistance to rapamycin (Fig. 8A). Combined deletion of *GCN2*/*GLN*3 or *GCN4*/*GLN3* resulted in further resistance that was comparable to the level seen in a dominant *TOR1* mutant (*TOR1-S1972I*), which does not bind the Fpr1p/rapamycin complex (Fig. 8A) (165-166). Therefore, the combined deletions prevent blockade of


A
WT
gcn2Δ
gcn4Δ
gln3Δ
gcn2Δ gln3Δ
gcn4Δ gln3Δ
YPD
MSX
Rap
3-AT


B
GCN2
gcn2Δ
C
3AT
Rap
C
3AT
Rap
eIF2α~P
eIF2α

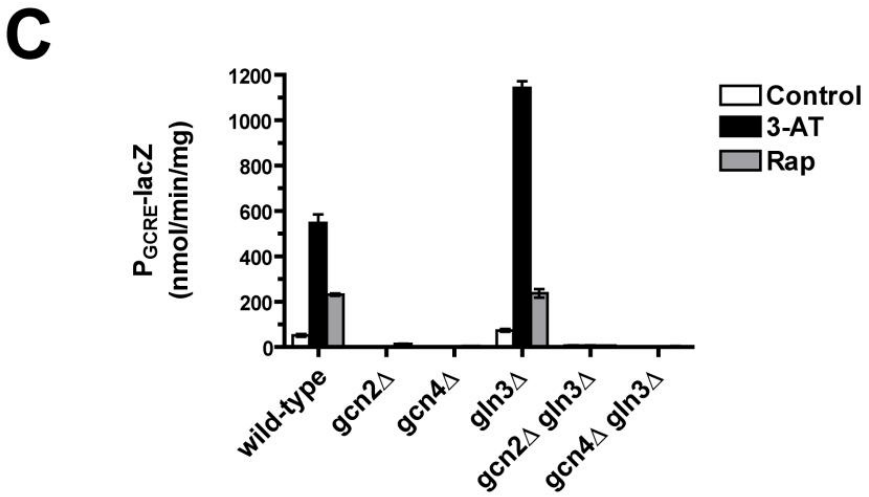
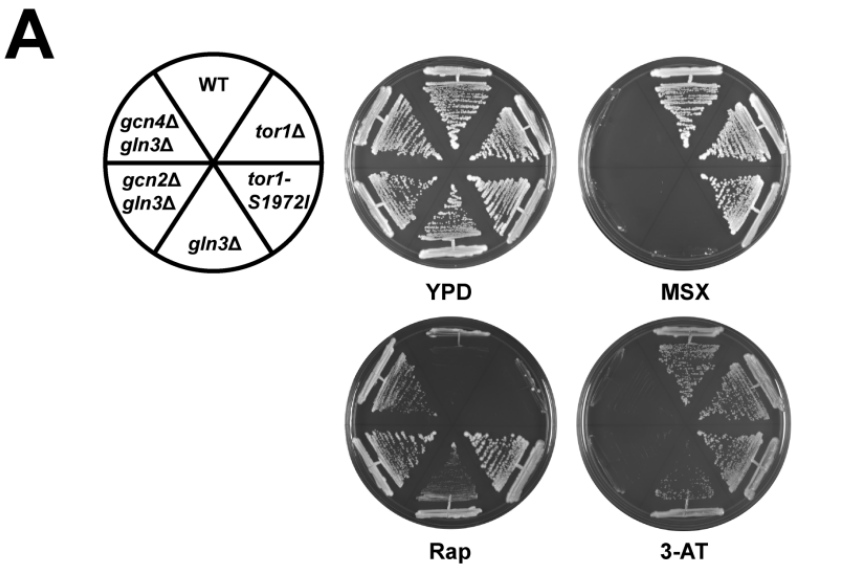
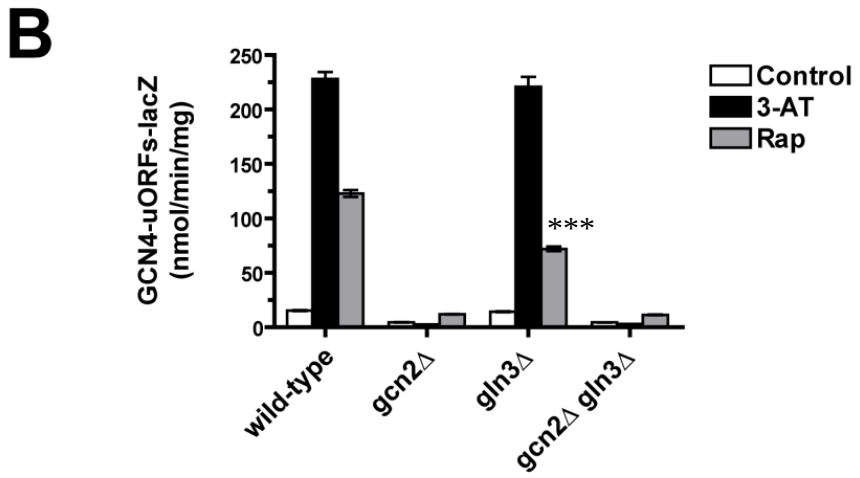

C
P GCRE-lacZ (nmol/min/mg)
1200
1000
800
600
400
200
0
Control
3-AT
Rap
wild-type
gcn2Δ
gcn4Δ
gln3Δ
gcn2Δ gln3Δ
gcn4Δ gln3Δ

Figure 7. *GCN2* is required for induced Gcn4p transcriptional activity in response to rapamycin or 3-AT treatment. (**A**) Growth of prototrophic yeast strains were determined by streaking onto YPD agar medium (YPD) or YPD containing 2 mM MSX (MSX) or 200 nM rapamycin (RAP), as indicated. Strains were also streaked on synthetic complete (SC) media (lacking histidine) containing 30 mM 3-AT (3-AT). (**B**) Wild-type and *gcn2*Δ cells were treated with 10 mM 3-AT (3AT), 200 nM rapamycin (Rap), or no stress (C, control) for 1 hour, and the levels of eIF2α phosphorylated specifically at serine-51, or total eIF2α, were measured by immunoblot analyses. (**C**) Yeast cells containing a P_{GCRE}-*lacZ* reporter plasmid including the consensus GCRE were treated with 3-AT or rapamycin, as listed in the legend. The average β-galactosidase activity from three independent cultures ± S.E. is shown.


A
WT
tor1Δ
tor1-S1972I
gln3Δ
gcn2Δ gln3Δ
gcn4Δ gln3Δ
YPD
MSX
Rap
3-AT


B
GCN4-uORFs-lacZ (nmol/min/mg)
250
200
150
100
50
0
***
Control
3-AT
Rap
wild-type
gcn2Δ
gln3Δ
gcn2Δ gln3Δ

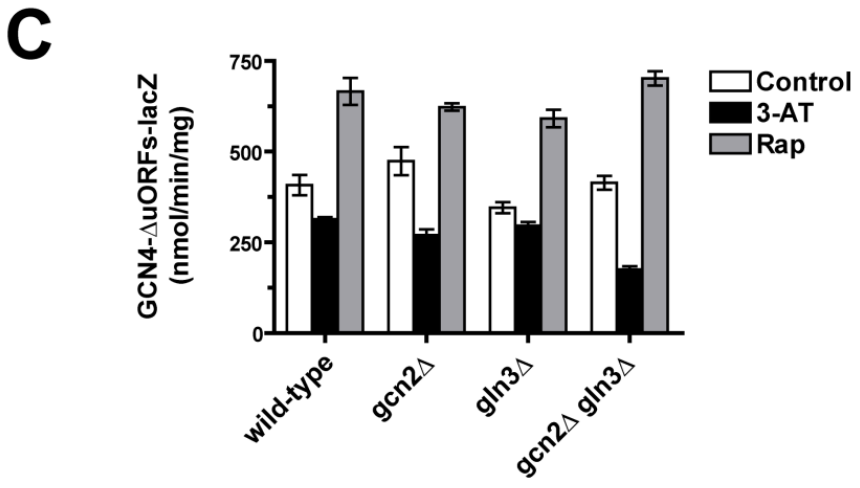

C
GCN4-ΔuORFs-lacZ (nmol/min/mg)
750
500
250
0
Control
3-AT
Rap
wild-type
gcn2Δ
gln3Δ
gcn2Δ gln3Δ

Figure 8. Loss of the GAAC renders cells growth resistant to rapamycin. (**A**) Wild-type cells, or mutant cells containing the indicated gene mutations, were streaked on YPD agar plates containing 200 nM rapamycin (Rap), 2 mM L-methionine sulfoximine (MSX), or no stress treatment (YPD). As a control, strains were also grown on synthetic complete media lacking histidine and lacking histidine and containing 30 mM 3-amino-triazole (3-AT). A *GCN4-lacZ* reporter plasmid (*GCN4*-uORFs-*lacZ*) with (**B**) or without (*GCN4*-ΔuORFs-*lacZ*) uORFs (**C**) was introduced into wild-type cells (WY837), or cells deleted for *GCN2* (WY838), *GLN3* (WY840), or *GCN2* and *GLN3* (WY841), as indicated. Cells were treated with 3-AT or rapamycin for 6 hours, and β-galactosidase activity was measured from whole cell extracts. ***, denotes a significant reduction ($p < 0.001$) in β-galactosidase activity in *gln3*Δ cells treated with rapamycin compared to wild-type cells treated with rapamycin. The average β-galactosidase activity from three independent cultures ± S.E. is shown.

TOR function by rapamycin, even in the presence of the functional *TOR2* locus. These results support the idea that the two major nutritional stress pathways can work in synergy, with both *GCN2* and *GCN4* contributing to gene expression mediated by the TOR signaling pathway.

II. Rapamycin induces Gcn2p phosphorylation of eIF2α and Gcn4p-mediated transcription

Treatment of the wild-type strain with either 3-AT or rapamycin increased Gcn2p-dependent phosphorylation of eIF2α and *GCN4* translational control, as measured by a *lacZ* reporter fused to the 5'-leader of the *GCN4* mRNA (Figs. 7B and 8B) (14-16). Deletion of *GCN2* blocked *GCN4* expression in response to either stress, while *gln3*Δ cells showed a modest, but significant ($p < 0.001$), decrease in *GCN4* expression in response to rapamycin exposure (Fig. 8B). Consistent with the idea that the GAAC regulates translation via uORFs in the 5'-leader of the *GCN4* mRNA, high levels of *GCN4* expression were measured in cells containing a similar *lacZ* reporter devoid of the *GCN4* uORFs independent of the stress treatment (Fig. 8C).

Importantly, elevated eIF2α phosphorylation and *GCN4* translation triggered increased Gcn4p-directed transcription as measured by a *lacZ* reporter containing a minimal *CYC1* promoter containing consensus Gcn4p-binding sites (GCREs) (Fig. 7C). Gcn4p transcriptional activity was blocked by deletion of either *GCN4* or *GCN2* (Fig. 7C). Interestingly, cells devoid of *GLN3* increased Gcn4p transcriptional activity by an additional 2-fold in response to 3-AT, suggesting an underlying

compensatory system in which loss of portions of the TOR pathway further enhance the GAAC.

III. Gcn4p is a major contributor to *TOR*-mediated gene expression

1. Changes in the yeast transcriptome following treatment with 3-AT or rapamycin

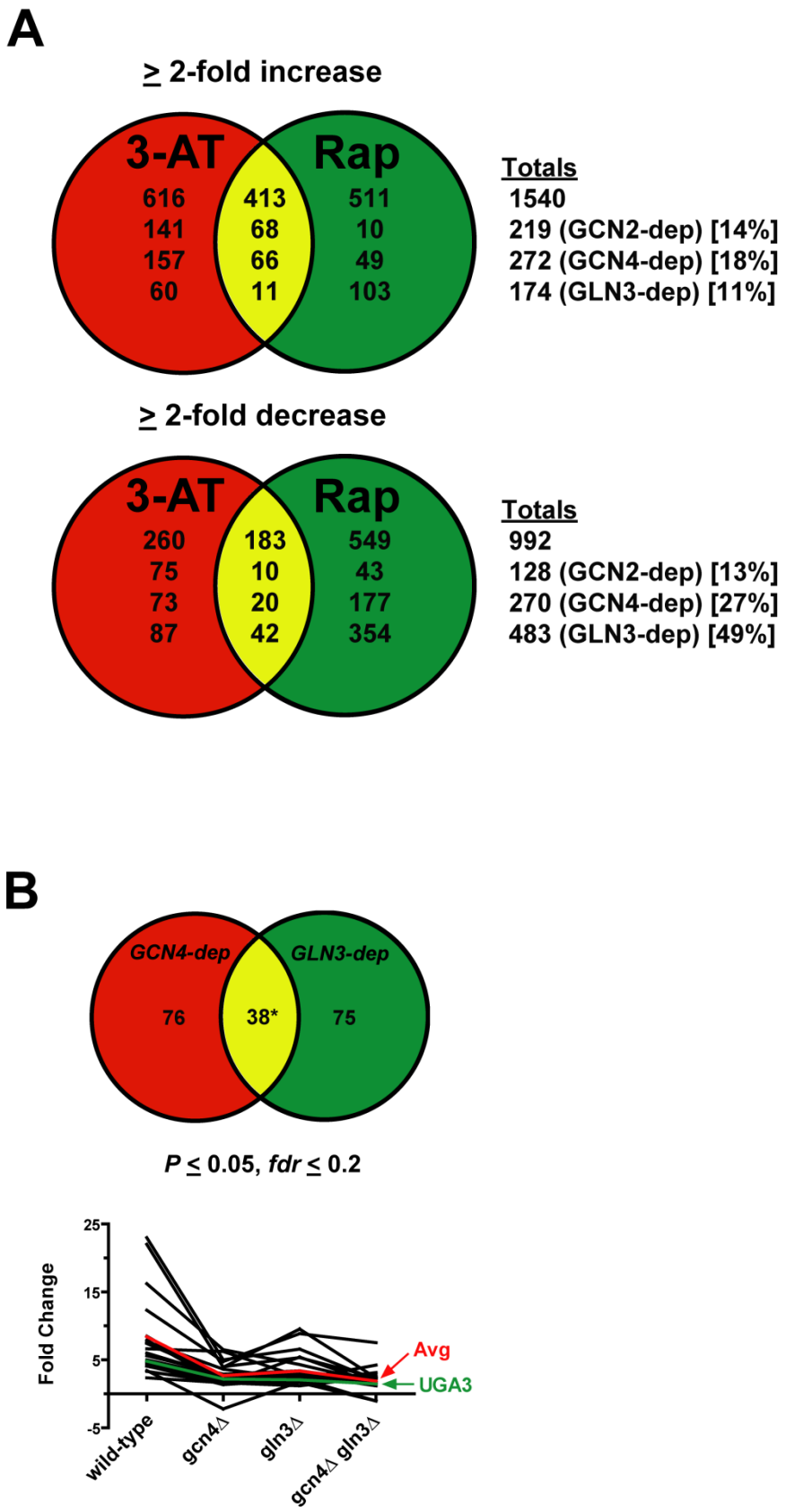
To address the roles of Gcn4p and Gln3p in gene expression induced by either 3-AT or rapamycin treatment, we carried out whole genome transcriptional profiling experiments using Affymetrix GeneChips to measure mRNA levels in an isogenic set of strains (Fig. 9 and Table 1). Cells deleted for *GCN2*, *GCN4* or *GLN3*, or their combined mutations (*gcn2*Δ *gln3*Δ and *gcn4*Δ *gln3*Δ), were cultured for 1 hour in the presence of either 10 mM 3-AT or 200 nM rapamycin and compared to a wild-type strain that was similarly treated. Of the genes that were defined by probe sets, a significant ($p \leq 0.05$, FDR ≤ 0.2) change in expression was observed in >5100 probe sets (out of 9275 on the S98 array) representing 4052 uniquely annotated genes. A ≥ 2-fold increase was observed in 2532 probe sets representing 2103 uniquely annotated genes (total of increases and decreases; Fig. 10A). This indicates that a substantial portion of the yeast transcriptome, ~30% of the encoded genes, is regulated by either amino acid starvation or rapamycin-mediated inactivation of the TOR pathway.

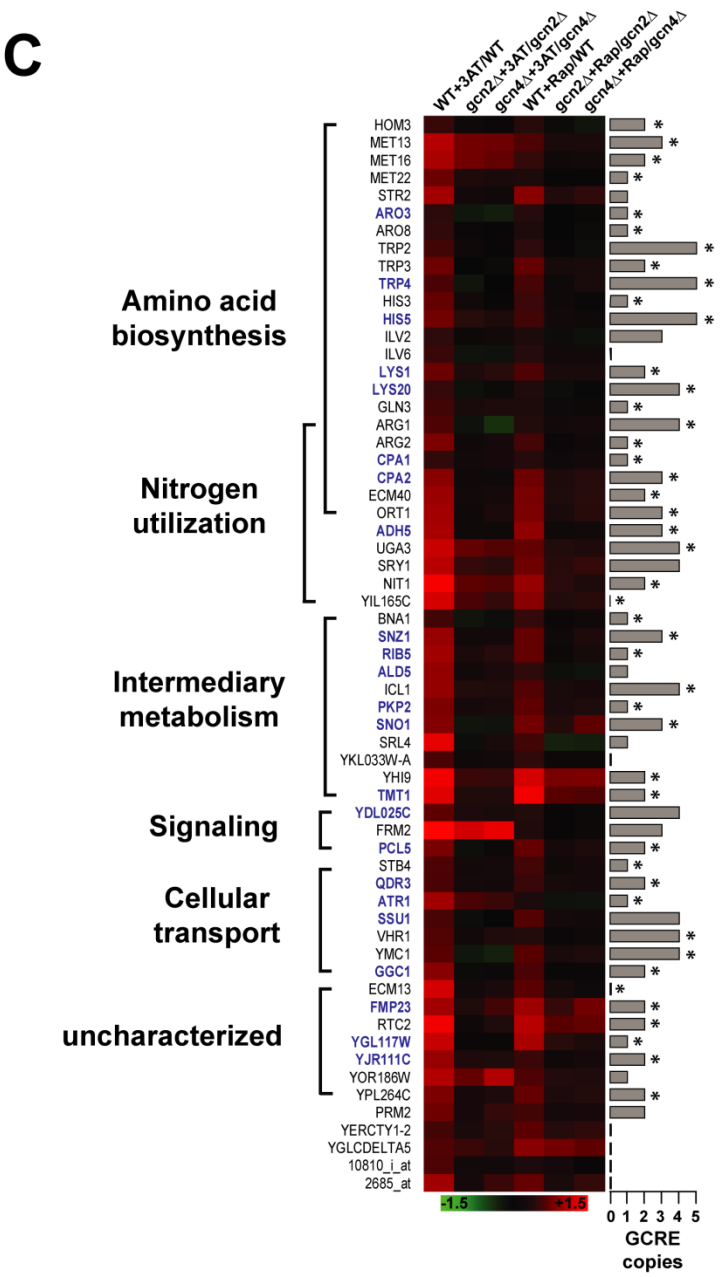
2. Genes induced by 3-AT

In cells exposed for 1 h to 10 mM 3-AT, there was ≥ 2-fold increased expression of 1029 genes (700 annotated, Table 4), which correlates well with those

		Treatment		
Strain	**Genotype**	**Control**	**3AT**	**Rap**
WY798	WT	1	2	3
WY799	gcn2Δ	4	5	6
WY857	gcn4Δ	7	8	9
WY858	gln3Δ	10	11	12
WY859	gcn2Δ gln3Δ	13	14	15
WY860	gcn4Δ gln3Δ	16	17	18

Figure 9. Design of whole genome transcriptional profiling experiments in yeast. The microarray experiment included 18 treatment groups involving six isogenic yeast strains and three treatment conditions. Wild-type (WT), and relevant gene deletions are indicated. Strains were grown in SC media lacking histidine and treated with 10 mM 3-AT (3AT), 200 nM rapamycin (Rap), or no stress (Control) for 1 hour.


A
≥ 2-fold increase
3-AT
Rap
616
141
157
60
413
68
66
11
511
10
49
103
Totals
1540
219 (GCN2-dep) [14%]
272 (GCN4-dep) [18%]
174 (GLN3-dep) [11%]
≥ 2-fold decrease
3-AT
Rap
260
75
73
87
183
10
20
42
549
43
177
354
Totals
992
128 (GCN2-dep) [13%]
270 (GCN4-dep) [27%]
483 (GLN3-dep) [49%]
B
GCN4-dep
GLN3-dep
76
38*
75
P ≤ 0.05, fdr ≤ 0.2
Fold Change
25
15
5
-5
Avg
UGA3
wild-type
gcn4Δ
gln3Δ
gcn4Δ gln3Δ


C
WT+3AT/WT
gcn2Δ+3AT/gcn2Δ
gcn4Δ+3AT/gcn4Δ
WT+Rap/WT
gcn2Δ+Rap/gcn2Δ
gcn4Δ+Rap/gcn4Δ
Amino acid biosynthesis
Nitrogen utilization
Intermediary metabolism
Signaling
Cellular transport
uncharacterized
HOM3
MET13
MET16
MET22
STR2
ARO3
ARO8
TRP2
TRP3
TRP4
HIS3
HIS5
ILV2
ILV6
LYS1
LYS20
GLN3
ARG1
ARG2
CPA1
CPA2
ECM40
ORT1
ADH5
UGA3
SRY1
NIT1
YIL165C
BNA1
SNZ1
RIB5
ALD5
ICL1
PKP2
SNO1
SRL4
YKL033W-A
YHI9
TMT1
YDL025C
FRM2
PCL5
STB4
QDR3
ATR1
SSU1
VHR1
YMC1
GGC1
ECM13
FMP23
RTC2
YGL117W
YJR111C
YOR186W
YPL264C
PRM2
YERCTY1-2
YGLCDELTA5
10810_i_at
2685_at
-1.5
+1.5
0 1 2 3 4 5
GCRE copies

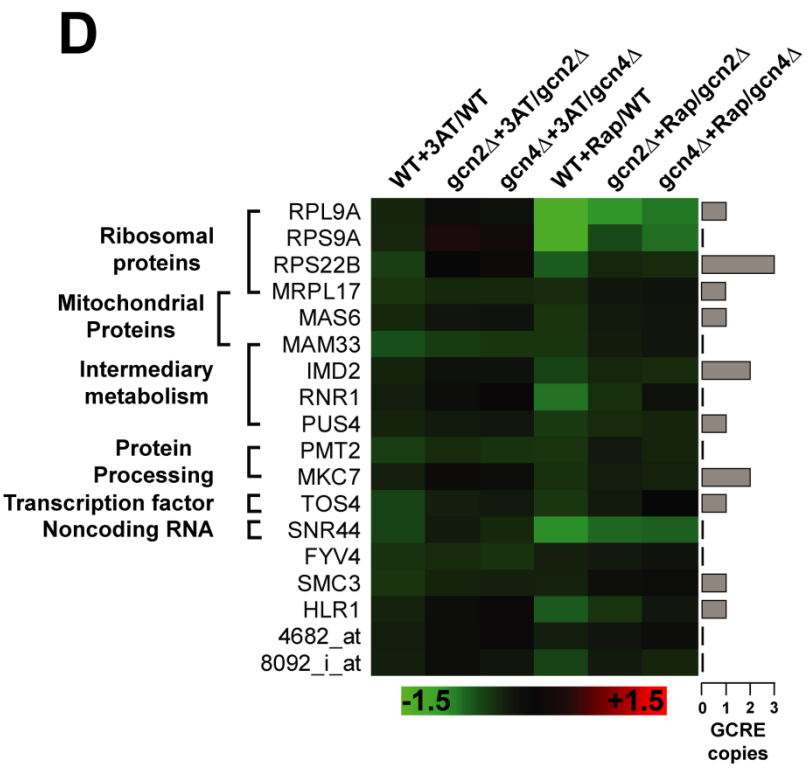
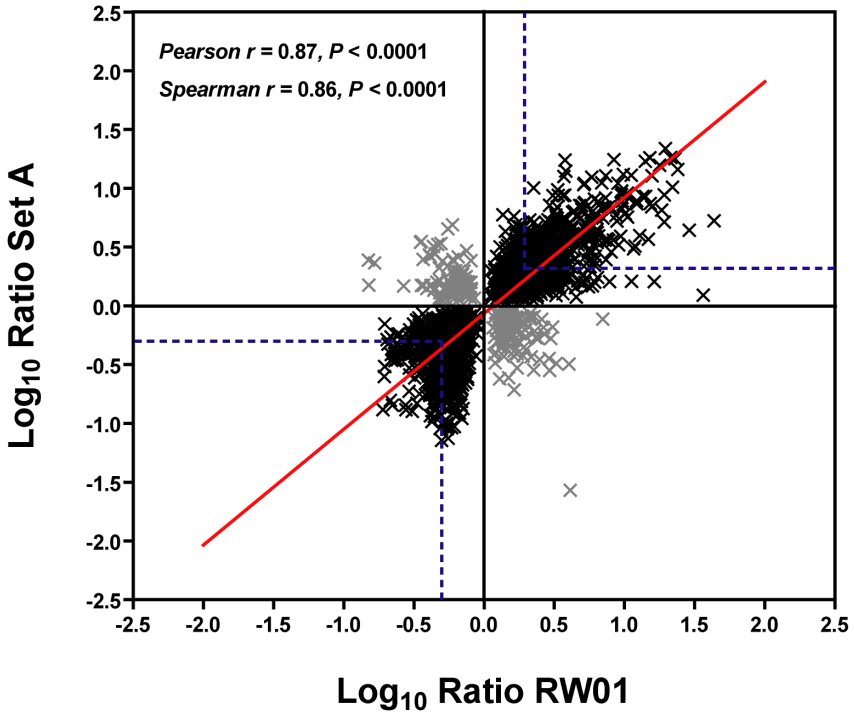


Figure 10. The role of GAAC and TOR in the changes of the yeast transcriptome following treatment with rapamycin or 3-AT. (**A**) Venn diagrams illustrating the number of genes whose encoded mRNAs require *GCN2*, *GCN4*, or *GLN3* for a 2-fold change in expression following 3-AT or rapamycin treatment. Red indicates those gene transcripts changed only by 3-AT, green only in response to rapamycin treatment, and yellow by both stress treatments. The total number of transcripts changed for each mutant and the percentage of the total number of transcripts changed in the wild-type strain are indicated at the right of the figure. (**B**) (*Top*) Venn diagram illustrating the number of genes whose encoded mRNAs require *GCN4* or *GLN3* for a ≥ 2-fold increase following rapamycin exposure. Red indicates those gene transcripts requiring only *GCN4*, green requiring only *GLN3*, and yellow requiring both transcriptional regulators. (*Bottom*) Rapamycin-induced fold-changes for gene transcripts in wild-type, *gcn4*Δ, *gln3*Δ, or *gcn4*Δ *gln3*Δ are plotted individually in black. The average fold-change for all gene transcripts is shown in red and the fold-change values for *UGA3* mRNA are highlighted in green. Heat maps illustrate the levels of gene transcripts which require *GCN4* for increased (**C**) or decreased (**D**) expression following treatment with 3-AT or rapamycin. Genes in the Gcn4p activation or repression core are listed, along with their general biological functions. The legend at the bottom of the heat maps illustrates the changes in transcript levels between the paired samples listed at the top of each map. The number of GCREs present in each gene promoter region is represented to the right of the heat maps. In Fig. 10C, the asterisks indicate promoters reported to bind Gcn4p in chromatin immunoprecipitation (ChIP-chip) experiments (156), and genes highlighted in *blue* were reported to have increased transcription in medium supplemented with secondary group B nitrogen compounds (159).

genes (both identity and magnitude of induction) reported to be induced by treatment with 100 mM 3-AT (79) (Fig. 11A). A histogram depicting the distribution of the fold-changes for these genes is shown in Fig. 11B. Of the genes induced by 3-AT treatment, 209, 223, and 71 were dependent on *GCN2*, *GCN4*, or *GLN3*, respectively (Table 4 and supplemental Table S2 (153)). A large portion of the GAAC-dependent genes activated by 3-AT are involved in amino acid metabolism, a major focus for Gcn4p (18,79) (supplemental Table S2, (153)). Using this ≥ 2-fold induction criteria, Gcn4p was required for increased expression of 46 amino acid biosynthetic genes, participating in the synthesis of each of the amino acids, with the exception of proline. In this later case, Gcn4p was required for a 1.6-fold increase in expression of *PRO*2 ($p < 0.001$), encoding γ-glutamyl phosphate reductase (catalyzing the second step in the biosynthesis of proline), which although significant was below the ≥ 2-fold threshold. Related to this biosynthetic process, Gcn4p also induced genes involved vitamin metabolism (7-targeted genes involved in the biosynthesis of pyridoxal phosphate, NAD, folate, coenzyme A, or riboflavin), intermediary metabolism (12 genes) and transport processes (5 genes) [highlighted in *bold red* in supplemental Table S2, (153)]. It is noteworthy that Gcn4p also plays an important role in nitrogen utilization, targeting 12 genes in this functional category. The mechanistic role of Gcn4p in nitrogen utilization will be explored further below.

Among the genes requiring either *GCN2* or *GCN4* for full induction, a significant majority (77%, Representation Factor = 37.4) of these required both regulators of the GAAC, supporting the idea that Gcn2p is a central upstream activator of the Gcn4p. A subset of these (35 genes) were found to be significantly

A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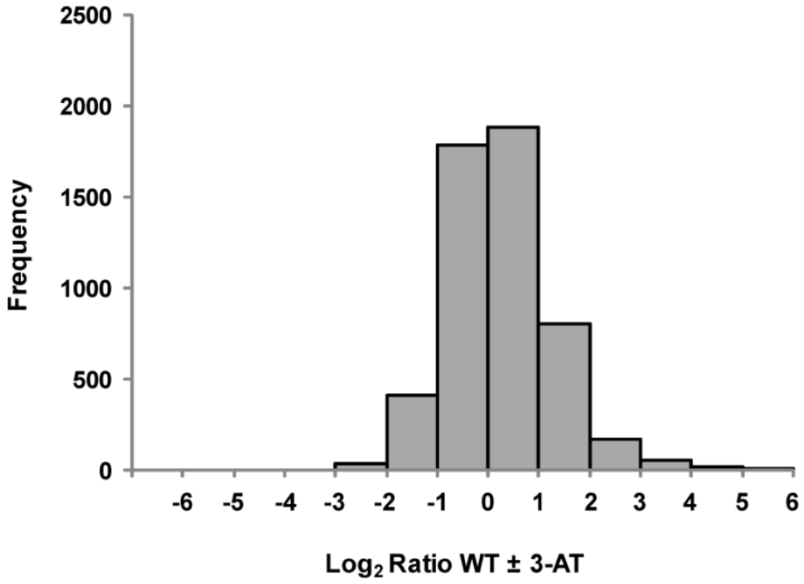
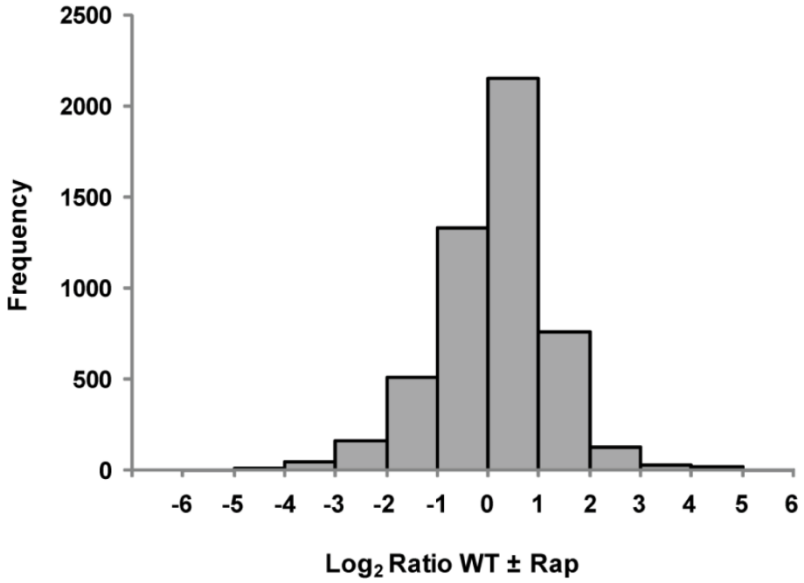
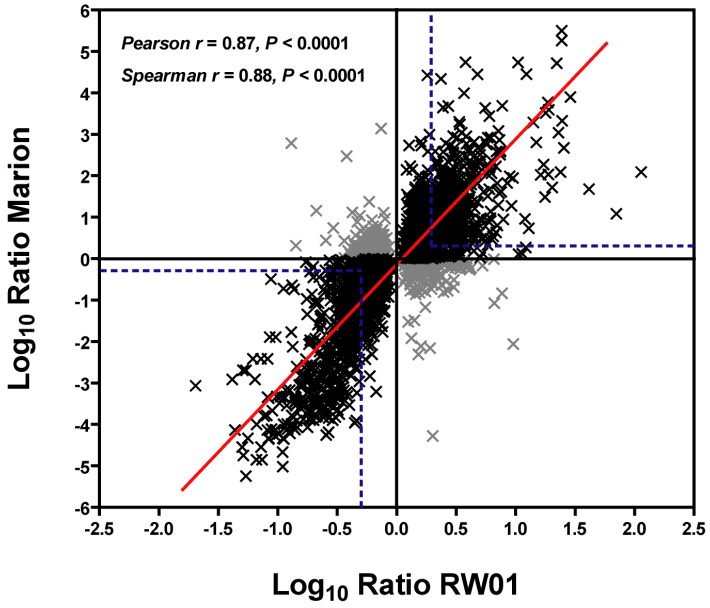
Spearman r = 0.86, P < 0.0001
Log10 Ratio Set A
Log10 Ratio RW01
2.5
2.0
1.5
1.0
0.5
0.0
-0.5
-1.0
-1.5
-2.0
-2.5
-2.5
-2.0
-1.5
-1.0
-0.5
0.0
0.5
1.0
1.5
2.0
2.5

B

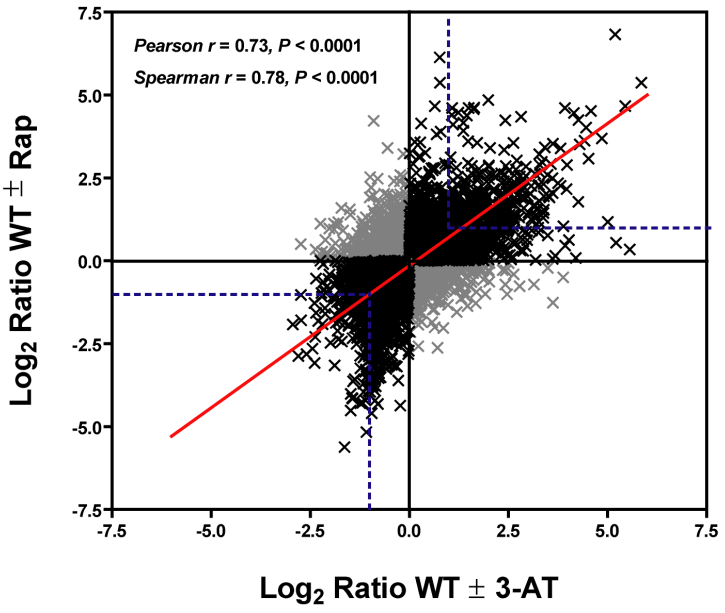

2500
2000
1500
1000
500
0
Frequency
-6 -5 -4 -3 -2 -1 0 1 2 3 4 5 6
Log2 Ratio WT ± 3-AT


2500
2000
1500
1000
500
0
Frequency
-6 -5 -4 -3 -2 -1 0 1 2 3 4 5 6
Log2 Ratio WT ± Rap

C


Pearson r = 0.87, P < 0.0001
Spearman r = 0.88, P < 0.0001
Log10 Ratio Marion
Log10 Ratio RW01
6
5
4
3
2
1
0
-1
-2
-3
-4
-5
-6
-2.5
-2.0
-1.5
-1.0
-0.5
0.0
0.5
1.0
1.5
2.0
2.5

D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Spearman r = 0.78, P < 0.0001
Log2 Ratio WT ± Rap
Log2 Ratio WT ± 3-AT
7.5
5.0
2.5
0.0
-2.5
-5.0
-7.5
-7.5
-5.0
-2.5
0.0
2.5
5.0
7.5

Figure 11. Comparative analysis of genes induced by 3-AT or rapamycin treatment. (**A**) A comparative analysis of genes induced or repressed by 3-AT in this study (RW01) with those in Set A of Natarajan et al., 2001 (79). A Log_{10} scatter plot is shown displaying all probe sets representing transcripts which showed a significant change ($p \leq 0.05$, FDR ≤ 0.2) in expression following treatment with 10 mM 3-AT for 1 hour. Transcripts which correlated positively (2103 total) or negatively (206 total) are indicated by black and grey stars, respectively. A trend line is shown in red for those transcripts which displayed a significant positive correlation (Pearson $r = 0.87$, $p < 0.0001$, Spearman $r = 0.85$, $p < 0.0001$). Transcripts which changed by 2-fold or more ($-0.33 \leq Log_2 \geq 0.33$) are enclosed by blue dotted lines in upper right and lower left quadrants. (**B**) The distribution of the yeast transcriptome following treatment with 3-AT (*top panel*) or rapamycin (*bottom panel*) is depicted as a histogram indicating the number of genes that were significantly induced or repressed ($p \leq 0.05$, FDR ≤ 0.2). The number of probe sets representing transcripts at each value of Log_2 ratio are plotted. (**C**) A comparative analysis of genes induced or repressed by rapamycin in this study (RW01) with those in Marion et al., 2004 (134). A Log_{10} scatter plot is shown displaying all probe sets representing transcripts which showed a significant change ($p \leq 0.05$, FDR ≤ 0.2) in expression following treatment with 200 nM rapamycin for 1 hour. Transcripts which correlated positively (2390 total) or negatively (371 total) are indicated by black and grey stars, respectively. A trend line is shown in red for those transcripts which displayed a significant positive correlation (Pearson r = 0.87, $p < 0.0001$, Spearman $r = 0.88$, $p < 0.0001$). Transcripts which changed by 2-fold or more ($-0.33 \leq Log_{10} \geq 0.33$) are enclosed by blue dotted lines in upper right and lower left quadrants. (**D**) A comparative analysis of transcripts induced or repressed following treatment with 3-AT or rapamycin. A Log_2 scatter plot is shown displaying all probe sets representing mRNAs which showed a significant change ($p \leq 0.05$, FDR ≤ 0.2) in expression following treatment with 10 mM 3-AT or 200 nM rapamycin for 1 hour. Transcripts which correlated positively or negatively are indicated by black and grey stars, respectively. A trend line is shown in red for those transcripts which displayed a significant positive correlation (Pearson $r = 0.73$, $p < 0.0001$, Spearman $r = 0.78$, $p < 0.0001$). Transcripts whose levels changed by 2-fold or more ($-1.0 \leq Log_2 \geq 1.0$) are enclosed by *blue* dotted lines in upper right and lower left quadrants.

TABLE 4. Summary of gene expression profiling experiments

Treatment	Dependence[b]	Number of probe sets[a]			
		Increase	≥ 2X increase	Decrease	≥ 2X decrease
3-AT	wild-type	2186	**1029**[c]	1521	**443**
3-AT	*GCN2*-dep	541	**209**	319	**85**
3-AT	*GCN4*-dep	579	**223**	333	**93**
3-AT	*GLN3*-dep	383	**71**	289	**129**
Rapamycin	wild-type	2236	**924**	1525	**732**
Rapamycin	*GCN2*-dep	177	**78**	93	**53**
Rapamycin	*GCN4*-dep	395	**115**	397	**197**
Rapamycin	*GLN3*-dep	526	**114**	596	**396**

[a]Number of probe sets representing transcripts that increased or decreased significantly ($p \leq 0.05$, FDR ≤ 0.2) following treatment with 3-AT or rapamycin, and the number of these significant probe sets in which the change was at least 2-fold.
[b]Transcripts whose expression is dependent on *GCN2*, *GCN4*, or *GLN3*, identified as those showing a $\geq$ 2-fold (induced genes) or significant change (repressed genes) in the comparison between wild-type + treatment vs. either *gcn2*Δ + treatment, *gcn4*Δ + treatment or *gln3*Δ + treatment.
[c]Probe sets highlighted in *bold* are described in supplemental Table S2, (153).

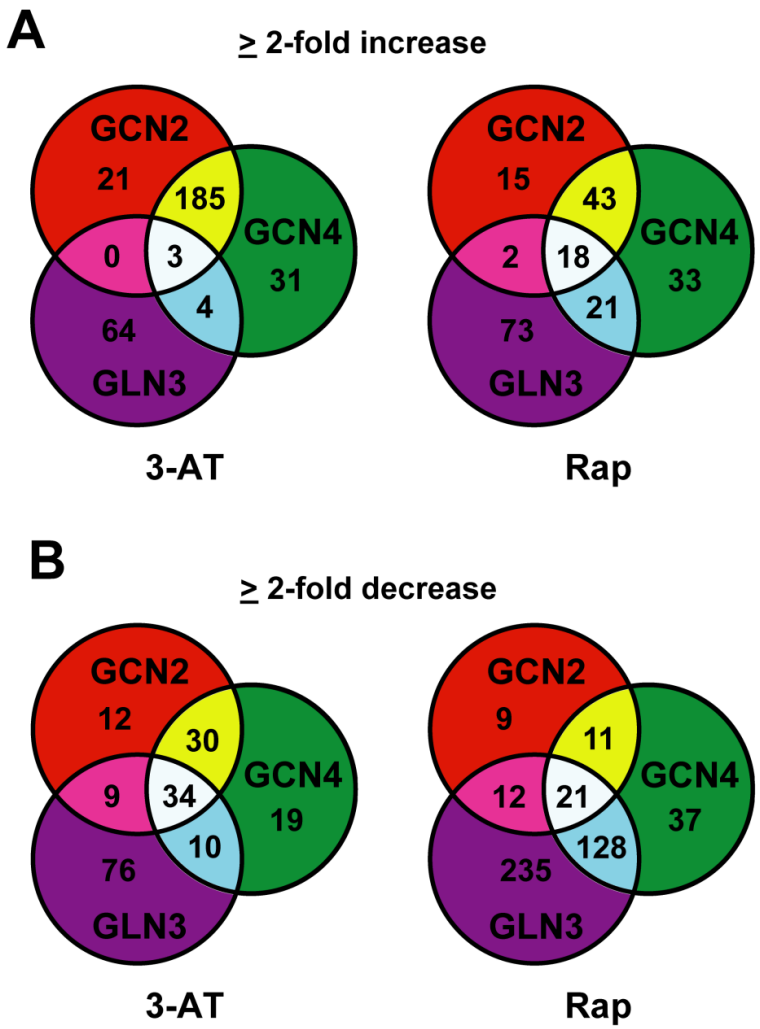


Figure 12. Requirements for *GCN2*, *GCN4*, and *GLN3* for changes in gene expression in response to 3-AT or rapamycin treatment. Venn diagrams illustrating the number of genes whose encoded mRNAs require *GCN2*, *GCN4*, or *GLN3*, as indicated, for a ≥ 2-fold increase (**A**) or ≥ 2-fold decrease (**B**) in expression following 3-AT or rapamycin treatment. The numbers of transcripts requiring two or all three of these regulatory genes for full induction or repression are indicated inside the colored overlapping regions.

more dependent on *GCN4*, as defined by those transcripts whose expression was increased ≥ 2-fold in wild-type cells treated with 3-AT compared to either the *gcn2*Δ and *gcn4*Δ mutant strains (Fig. 12A, supplemental Table S2, (153)). This finding argues that even basal amounts of Gcn4p, i.e. the levels of the transcription factor expressed in the absence of Gcn2p phosphorylation of eIF2α can be important for directing the transcriptome during certain stresses. Among the GAAC-dependent genes, a smaller number of genes showed a greater requirement for *GCN2* compared to *GCN4* (21 genes) using the same criteria (Fig. 12A and supplemental Table S2, (153)). These genes include *ARO9* and *ARO10*, which are involved in a catabolism of aromatic amino acids and will be discussed further below.

The 71 genes requiring *GLN3* for induction in response to 3-AT did not overlap substantially with those requiring *GCN4*, with only 10% of the *GLN3*-dependent gene showing a requirement for *GCN4* (Fig. 12A and supplemental Table S2 (153)). The largest group of genes requiring *GLN3* for induction in response to 3-AT were uncharacterized genes (14), followed by those related to transport (5), transcription processes (5), and metabolism (3). Overall, these results support the idea that the TOR-targeted Gln3p is a significant contributor to changes in the transcriptome in response to amino acid deprivation, a finding consistent with the sensitivity of the *gln3*Δ strain to 3-AT (Fig. 7A). However in the case of 3-AT, Gln3p-targeted genes are largely distinct from those regulated by Gcn4p.

3. Genes repressed by 3-AT

Fewer genes were repressed by 3-AT treatment (443 repressed ≥2-fold) as compared to rapamycin, with a total of 93 genes displaying significant dependence on Gcn4p (Table 4 and Fig. 10A). Included among the genes requiring Gcn4p for repression are those involved in protein synthesis, ribosomal proteins and ribosomal biogenesis, and well as genes involved in protein folding, targeting and sorting (supplemental Table S2 (153)). It is also noteworthy that there was significant overlap among the Gcn4p-repressed genes and those reduced during the so-called environmental stress response (167) (105 out of 261 annotated genes, $p < 1.4x10^{-43}$). This suggests that Gcn4p-dependent repression can occur in response to diverse environmental stresses.

Gln3p contributes to repression of an even larger set of genes compared to Gcn4p (129 versus 93; Table 4), and these repressed genes are involved in the same processes as described above for Gcn4p. Of these genes, 44 genes require both Gcn4p and Gln3p, suggesting significant regulatory overlap between the genes repressed by the TOR and GAAC pathways (Fig. 12B and supplemental Table S2, (153)). We conclude that Gln3p can be a significant contributor to regulation of the transcriptome in response to amino acid starvation.

4. Genes induced by rapamycin

Treatment of cells with rapamycin resulted in 924 transcripts (695 annotated genes) being induced ≥ 2-fold, which correlates well with those reported by Marion et al.(134) (Fig. 11C). The distribution of the fold changes for these genes is shown in Fig. 11B. There is a moderate correlation (Pearson $r = 0.73$, Spearman $r = 0.78$) between

those genes regulated by rapamycin and those regulated by 3-AT (Fig 11D). Of these induced transcripts, 115 and 78 genes showed dependence for *GCN4* or *GCN2*, respectively (Fig. 10A and Table 4). Almost a third of the Gcn4p target genes did not show significant requirements for *GCN2*, supporting the idea that basal amounts of Gcn4p are significant contributors to the transcriptome in response to rapamycin, an idea noted above for 3-AT stress. The genes induced by Gcn4p in response to rapamycin can be divided into two groups. The first includes 57 genes that were also activated during 3-AT stress. We will refer to those genes requiring Gcn4p for induction in response to either 3-AT or rapamycin stress as the Gcn4p activation core (Fig. 10C and supplemental Table S3, (153)). As will be discussed further below, the core genes are involved in amino acid biosynthesis, nitrogen utilization, intermediary metabolism and cellular transport. The second group includes those Gcn4p-targed genes specifically induced in response to rapamycin, which were centered on the generation and utilization of energy-related compounds (11 genes) (Fig. 10A and supplemental Table S2, (153)). The significant differences between the genes induced by Gcn4p by 3-AT and rapamycin indicates that the GAAC transcriptional regulation can be tailored to meet specific stress arrangements.

The number of genes dependent on *GCN4* for induced expression during rapamycin treatment was similar to those requiring *GLN3* (115 versus 114 in Table 4). This result demonstrates that Gcn4p has a major role in the induction of TOR-regulated genes, comparable to that of the known TOR effector, Gln3p. An analysis of genes requiring both Gcn4p and Gln3p for enhanced expression in response to rapamycin indicated that 38 were reduced by the loss of either transcriptional activator (Fig. 10B).

When *GCN4* and *GLN3* were deleted individually or in combination, 24 genes showed loss of induction in response to rapamycin stress, as illustrated in Fig. 10B. The average fold change for this collection of genes is illustrated in red. Many of these genes are suggested to carry out catabolic processes (Table 5 and supplemental Table S4, (153)), and we will explore further the coordinate regulation of genes involved in GABA utilization below. Paradoxically, among the 38 genes, 14 genes showed full or enhanced induction when both *GCN4* and *GLN3* were deleted, suggesting that there are compensatory mechanisms when both are ablated.

5. Genes repressed by rapamycin

Rapamycin treatment led to ≥ 2-fold repression of 732 genes, with 197 of these being dependent on *GCN4* (Fig. 10A and supplemental Table S4 (153)). The largest functional classes among the Gcn4p repressed genes are involved in ribosome biogenesis, including rRNA processing and modification, protein synthesis, transcription and nucleotide metabolism (supplemental Table S2, (153)). Although these genes overlap in their functional classes with those repressed during 3-AT, their identities are largely unique. There were 20 probe sets (Fig. 10A), representing 16 identifiable genes, that were repressed in response to either stress arrangement (Fig. 10D and supplemental Table S5, (153), Gcn4p repression core). These genes encode ribosomal proteins, as well as those involved in the mitochondria, intermediary metabolism, and protein processing. It is interesting to note that only 53 of the genes repressed by rapamycin required *GCN2* (Table 4). Thus as noted above for 3-AT, a large number of genes requiring Gcn4p for

regulation did not show significant requirements for *GCN2*, which would suggest an important role for basal amounts of Gcn4p for coordination of the transcriptome.

Gln3p contributes to repression of 396 probe sets (Table 4), which represent genes involved in the same processes as described above for Gcn4p. In fact 149 of the genes repressed by rapamycin require both Gcn4p and Gln3p (Fig. 10B). This finding reinforces the idea that Gcn4p is a major effector of the TOR pathway, with this GAAC regulator contributing to gene repression, as well as activation in response to rapamycin. Relief of repression of these genes may be at least one reason for the growth resistance of *gcn2*Δ, *gcn4*Δ, and *gln3*Δ cells to rapamycin treatment (Fig. 7A)

IV. The Gcn4p activation core (GAC) is induced by either 3-AT or rapamycin treatments

Analysis of the overlap between genes induced in response to either 3-AT or rapamycin identified 68 and 66 genes that required *GCN2* or *GCN4*, respectively, for full induction (Fig. 10A). By contrast, far fewer (11 genes) required *GLN3* for enhanced expression during both stress arrangements (Fig. 10A). As will be discussed further below, the two additional genes that were dependent on *GCN2*, but not *GCN4*, were *ARO9* and *ARO10*. We refer to the 66 probe sets (57 identifiable genes) induced by either 3-AT or rapamycin treatment by a mechanism requiring Gcn4p as the Gcn4 activation core (Fig. 10C and supplemental Table S3, (153)). A total of 53 genes in the Gcn4p activation core contain at least one copy of a GCRE, with promoters of 46 members previously shown to bind to Gcn4p in ChIP experiments (Fig. 10C) (156).

The Gcn4p activation core can be viewed as those genes whose expression is the foundation for GAAC-directed adaptation to different stress arrangements. A large portion of these core genes participate in amino acid metabolism (23 of 57 genes, $P \leq 2x10^{-18}$). Most represented among these biosynthetic genes are those involved in the aromatic amino acids (*ARO3*, *ARO8*, *TRP2*, *TRP3*, and *TRP4*) and arginine (*ARG2*, *ECM40* (*ARG7*), *CPA1*, *CPA2*, and *ARG1*) (Fig. 10C and supplemental Tables S2 and S3, (153)). Other major functional categories are genes involved in nitrogen utilization (11 genes), a point that will be further discussed below, and those contributing to cellular transport (7 genes). One of these metabolism-related genes is *BSC5* (*YNR069C*), which encodes the amino terminal portion of a coding region juxtaposed out-of-frame with a downstream ORF *YNR068C*. Together these combined ORFs encode a predicted polypeptide with homology to Bul1p, an ubiquitin-binding protein that is important for sorting of amino acid permeases and growth during stress conditions (168).

The Gcn4p activation core also includes three transcription factors: Gln3p, suggesting a regulatory intersection between the GAAC and TOR pathways; Stb4p, involved in histone deacetylation (169); and Uga3p, a zinc finger transcriptional activator of genes required for nitrogen assimilation from GABA (170). The coordination of Gcn4p and Gln3p in GABA catabolism will be further addressed below. Also part of the Gcn4p activation core is Pcl5p, a cyclin partner for the PHO85 protein kinase that is required for phosphorylation and subsequent feedback degradation of Gcn4p (74,171). Gcn4p induces the expression of two protein kinases genes, *PKP2* and *YDL025C* (*RTK1*). Pkp2p inhibits mitochondrial pyruvate complex, which catalyzes the oxidative decarboxylation of pyruvate to acetyl-CoA (172). This suggests that the GAAC restricts

carbon flux between glycolysis and the tricarboxylic acid cycle. Rtk1p has been tied to glucose homeostasis and is closely related to the Hrk1p protein kinase that regulates plasma membrane transporters (173-174). Finally, two TY elements and six probe sets which map to unannotated regions of the yeast genome are among the Gcn4p activation genes, suggesting that noncoding RNAs also contribute to Gcn4p-mediated stress responses.

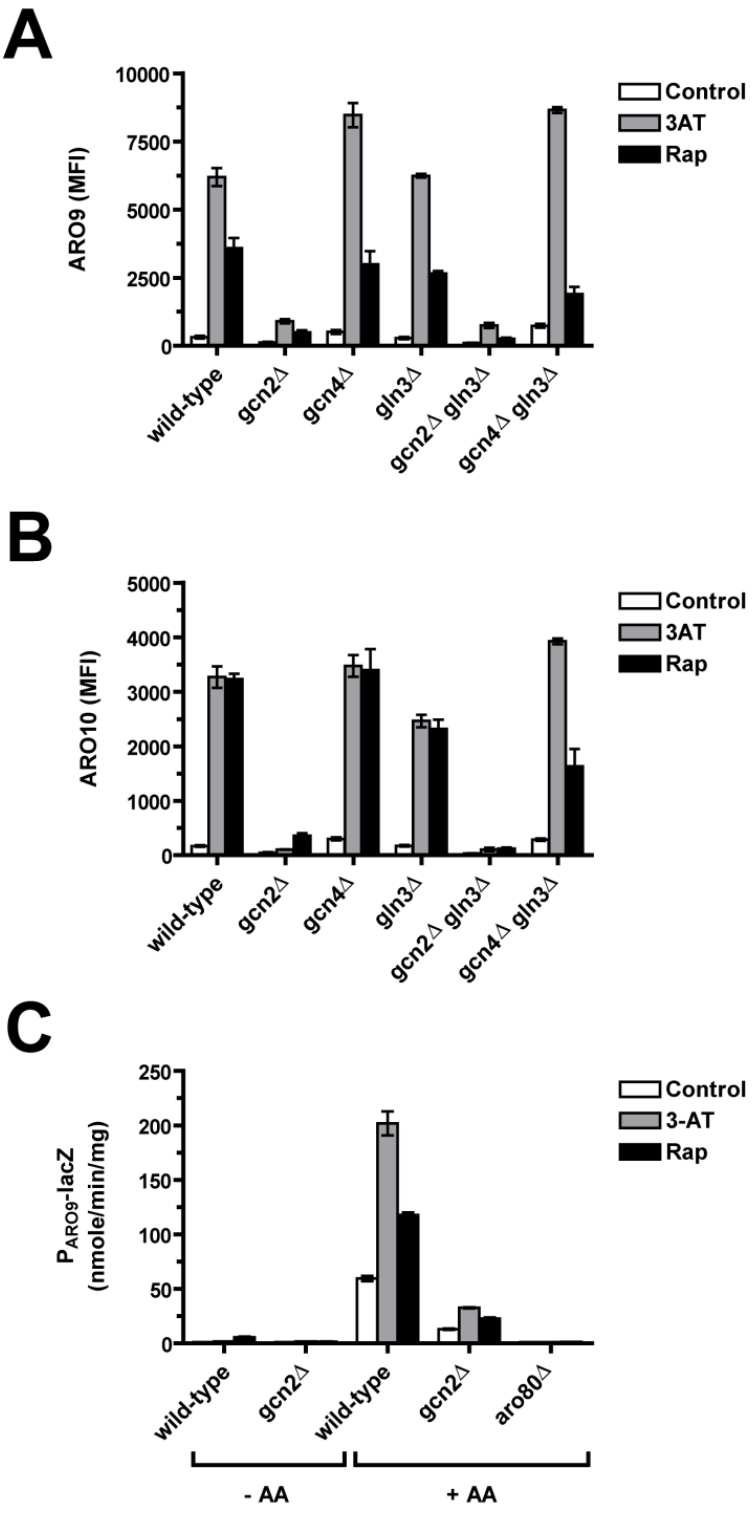
V. GAAC directs transcription of genes involved in assimilation of aromatic amino acids

Our transcriptome analysis suggested that the GAAC is important for directing gene transcription involved in nitrogen assimilation, as previously reported for the TOR pathway. Included among these genes are *ARO9* and *ARO10,* which are required for reclamation of nitrogen from aromatic amino acids via the Ehrlich pathway (175). As noted above, the microarray analysis showed increased expression of a small number of genes requiring Gcn2p in response to either 3-AT or rapamycin treatment, but these were largely independent of Gcn4p (Figs. 13A and B). Microarray measurements were independently confirmed by quantitative RT-PCR of *ARO9* and *ARO10* transcripts and additional select mRNAs (Fig. 14).

The transcription factor Aro80p is suggested to facilitate *ARO9* and *ARO10* transcription (176). We found that the levels of *ARO80* mRNA were not significantly changed during either stress condition or in response to deletion of *GCN2*, *GCN4*, or *GLN3* (supplemental Table S1, (153)). Additionally, we measured expression of the *ARO9* promoter (P_{ARO9}) fused to a *lacZ* reporter and found enhanced β-galactosidase

activity in response to either 3-AT or rapamycin, with the most robust increase during histidine starvation (Fig. 13C). Loss of *GCN2* resulted in a dramatic reduction in P_{ARO9}-*lacZ* activity in both control and stress conditions. This reporter assay supports the idea that the changes in *ARO9* mRNA levels measured in our microarray analysis were the result of GAAC-directed transcription at the P_{ARO9}. As expected, there was minimal P_{ARO9}-*lacZ* expression in cells deleted for *ARO80* (Fig. 13C). The induction of P_{ARO9}-*lacZ* by 3-AT or rapamycin was observed only when amino acids were supplemented to the minimal medium, suggesting that cells recognize starvation signals and induce the expression of key catabolic enzymes only when their respective substrates are available in the media (Fig. 13C).

These results suggest that the Gcn2p functions upstream or in a pathway parallel to Aro80p activation of genes that are important for catabolism of aromatic amino acids. Illustrating the key role of *ARO80* in aromatic catabolism, deletion of *ARO80* partially reduced growth in synthetic medium containing phenylalanine as the sole nitrogen source (Fig. 13D). No reduction in growth was found when the *aro80*Δ cells were grown in synthetic medium containing the nitrogen source ammonia, or in medium containing all twenty amino acids.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ARO9 (MFI)
10000
7500
5000
2500
0
Control
3AT
Rap
wild-type
gcn2Δ
gcn4Δ
gln3Δ
gcn2Δ gln3Δ
gcn4Δ gln3Δ
B
ARO10 (MFI)
5000
4000
3000
2000
1000
0
Control
3AT
Rap
wild-type
gcn2Δ
gcn4Δ
gln3Δ
gcn2Δ gln3Δ
gcn4Δ gln3Δ
C
P ARO9-lacZ
(nmole/min/mg)
250
200
150
100
50
0
Control
3-AT
Rap
wild-type
gcn2Δ
wild-type
gcn2Δ
aro80Δ
- AA
+ AA

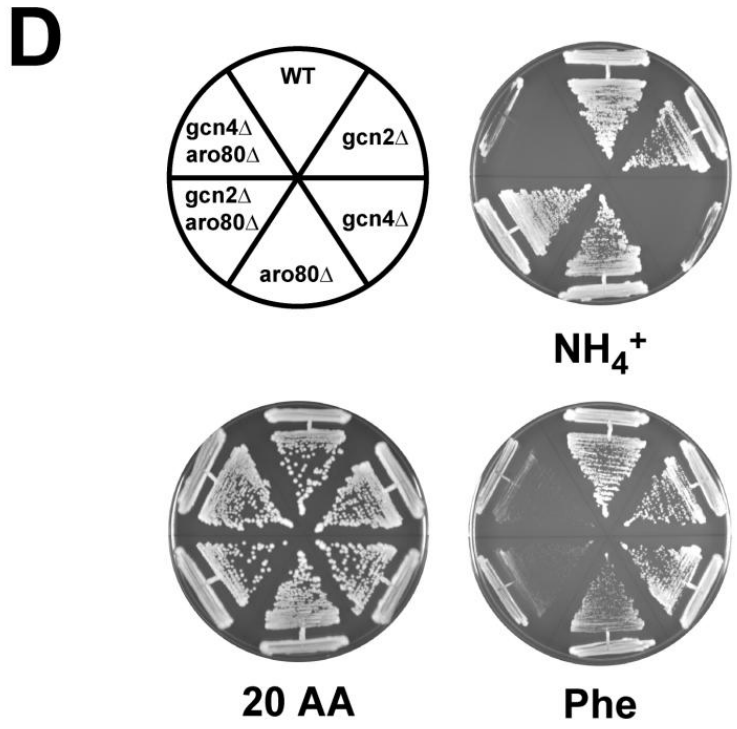


Figure 13. The GAAC and *ARO80* are required for expression of aromatic catabolism genes. Microarray measurements of *ARO9* (**A**) and *ARO10* (**B**) mRNA levels were measured in *gcn2*Δ, *gcn4*Δ, and *gln3*Δ cells, individually or in combination, which were treated with 3-AT or rapamycin, or no treatment (Control), as indicated. Transcript levels are plotted as mean fluorescence intensity (*MFI*). Changes in *ARO9* and *ARO10* mRNA levels were confirmed independently by quantitative RT-PCR in Fig. 14. (**C**) Wild-type (WY837), *gcn2*Δ (WY838), and *aro80*Δ (WY962) strains containing a P_{ARO9}-*lacZ* reporter plasmid were cultured in synthetic complete medium with (+AA) or without amino acids (-AA), as indicated, and treated with 3-AT or rapamycin, or not treated (Control) for 6 hours. β-galactosidase activity was measured from two independent cultures and is presented as the mean ± S.E. (**D**) Prototrophic strains were grown on synthetic agar plates containing either ammonia (NH_4^+), all twenty amino acids (20 AA), or phenylalanine (Phe) as the nitrogen source, as indicated.

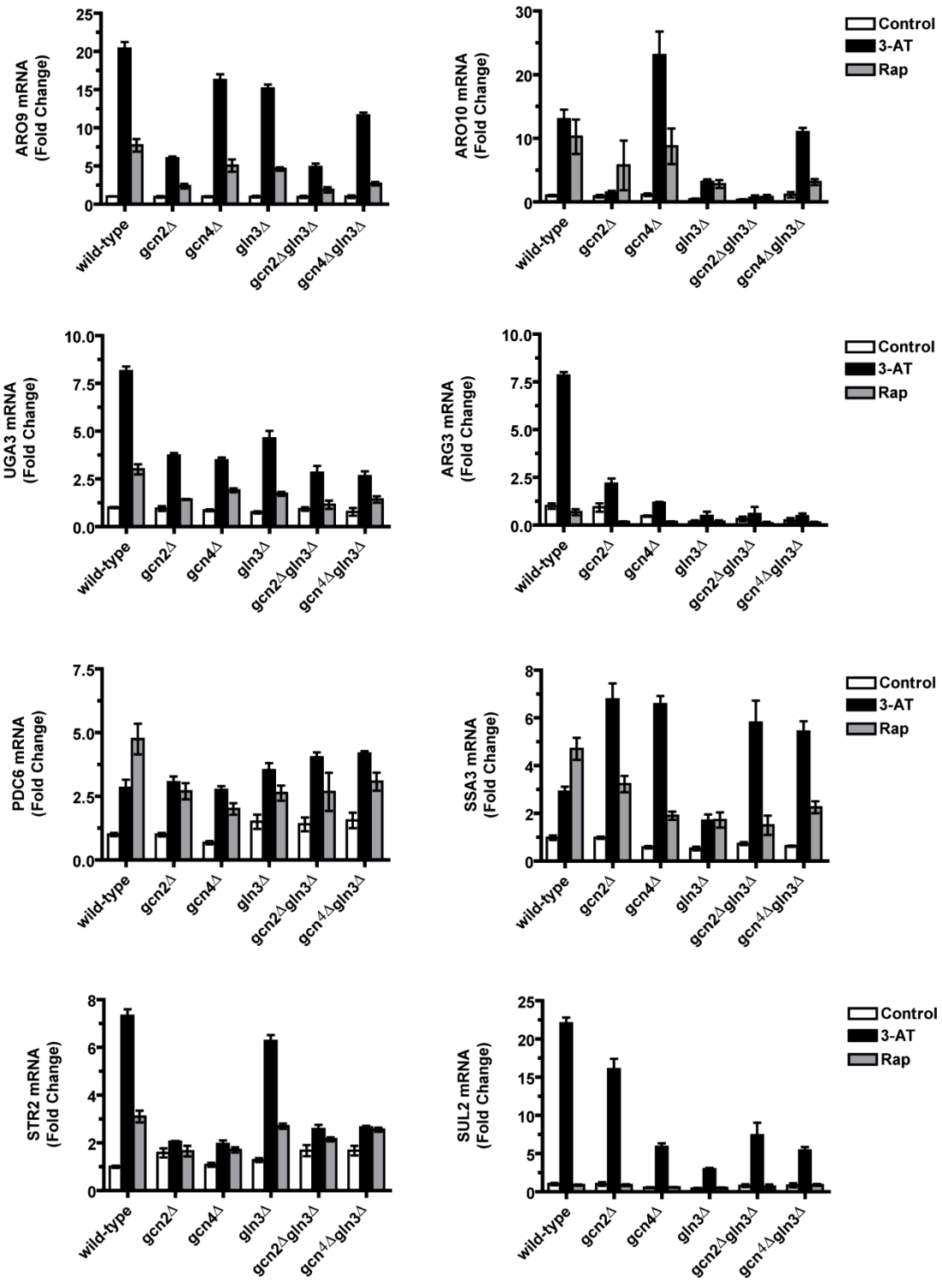

ARO9 mRNA (Fold Change)
ARO10 mRNA (Fold Change)
UGA3 mRNA (Fold Change)
ARG3 mRNA (Fold Change)
PDC6 mRNA (Fold Change)
SSA3 mRNA (Fold Change)
STR2 mRNA (Fold Change)
SUL2 mRNA (Fold Change)
Control
3-AT
Rap
wild-type
gcn2Δ
gcn4Δ
gln3Δ
gcn2Δgln3Δ
gcn4Δgln3Δ

Figure 14. qRT-PCR analysis of select transcripts in cells treated with 3-AT or rapamycin. qRT-PCR measurements of select mRNAs were carried out in wild-type (WY798), *gcn2*Δ (WY799), *gcn4*Δ (WY857), and *gln3*Δ (WY858) cells , deleted individually or in combination (WY859 and WY860). Cells were treated with 3-AT or rapamycin, or no treatment (Control), as indicated in the legend. Changes in transcript levels are expressed as fold-change relative to the untreated control in wild-type cells. The average fold-change from three to four independent experiments ± S.E. is shown.

Cells deleted for either *GCN2* or *GCN4* individually, or in combination with *ARO80* were also cultured in these media. All mutant strain combinations grew in medium containing all twenty amino acids. While deletion of *GCN2* alone did not elicit a growth defect in synthetic medium containing ammonia or phenylalanine as the nitrogen source, the combined *gcn2*Δ *aro80*Δ strain selectively displayed a severe growth defect in the phenylalanine medium (Fig.13D). Cells deleted for *GCN4* often have growth defects in minimal medium supplemented with ammonia but no amino acids, presumably due to their reduced capacity to synthesize amino acids. Interestingly, *gcn4*Δ cells grew to wild-type levels in synthetic medium supplemented with the nitrogen source phenylalanine (Fig. 13D). As observed for *gcn2*Δ mutants, introduction of *gcn4*Δ into the *aro80*Δ strain exacerbated the growth defect in the phenylalanine medium. These results indicate that the Gcn2p contributes to catabolism of aromatic amino acids via a pathway linked to Aro80p-directed transcription of *ARO9* and *ARO10*. As Gcn4p did not appear to be required for induced transcription of either *ARO9* or *ARO10*, Gcn4p may contribute to growth in media containing phenylalanine as a nitrogen source by coordinating amino acid biosynthesis and related metabolic pathways.

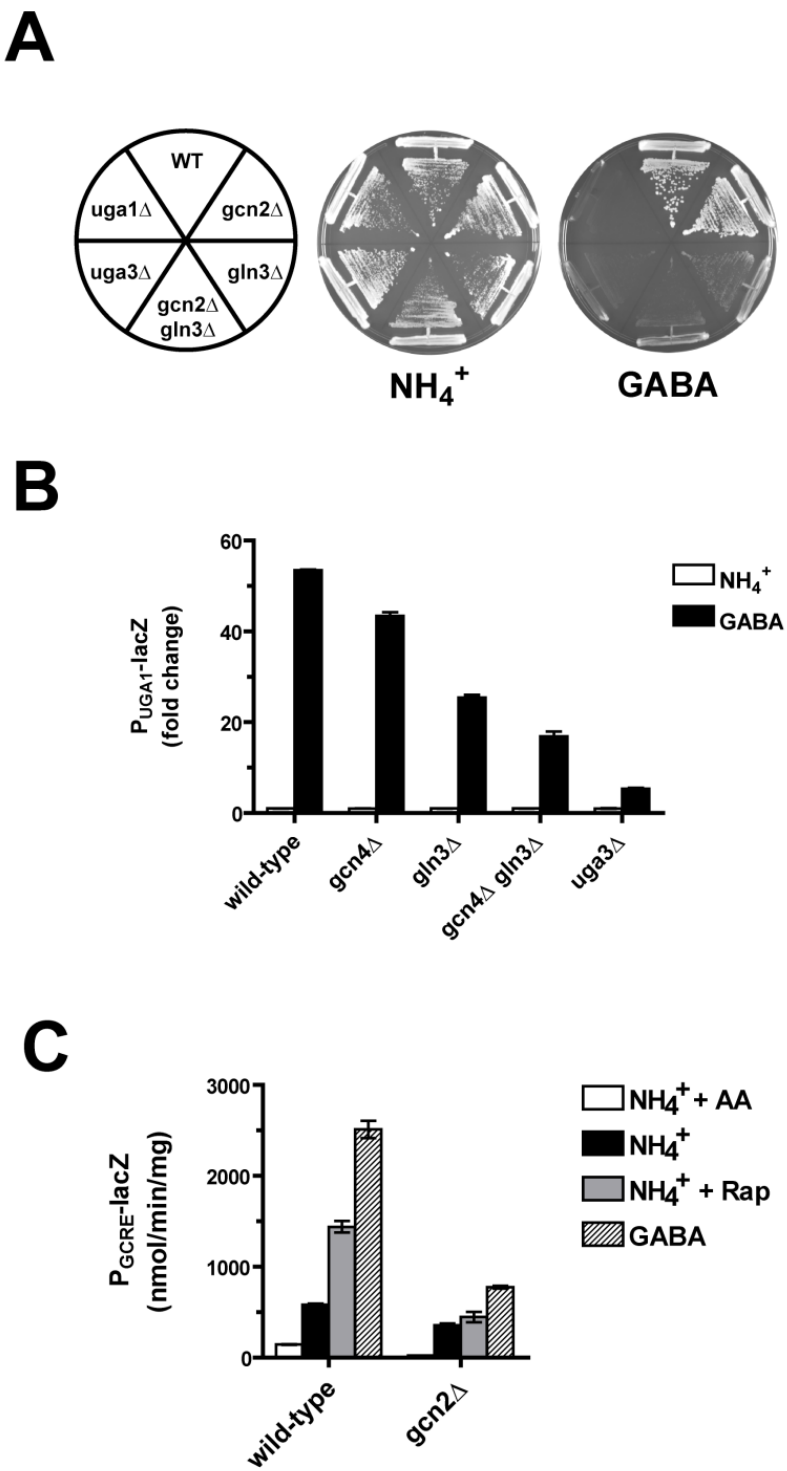
VI. Gcn4p and Gln3p stimulate GABA catabolism

One of the target genes in the Gcn4p activation core is *UGA3*, which also is jointly regulated by Gln3p (Fig. 10C and Table 5). Gcn4p is suggested to have an important role in nitrogen utilization, and *UGA3* encodes a transcription factor that directs expression of *UGA1*, *UGA2* and *UGA4*, which facilitate catabolism of GABA. Deletion of either *UGA3* or *UGA1* significantly blocked growth in minimal medium

TABLE 5. Genes co-regulated by GCN4p and GLN3p

Systematic Name[a]	Gene Name	Description
YLL055W	***YCT1***	High-affinity cysteine-specific transporter
YKL050C		Protein of unknown function
YKL218C	***SRY1***	3-hydroxyaspartate dehydratase, deaminates L-threo-3-hydroxyaspartate to form oxaloacetate and ammonia
YDL170W	***UGA3***	Transcriptional activator necessary for gamma-aminobutyrate (GABA)-dependent induction of GABA genes
YIR017C	*MET28*	Transcriptional activator in the Cbf1p-Met4p-Met28p complex
YIL165C		Putative protein of unknown function
YIL164C	***NIT1***	Nitrilase
YHR029C	*YHI9*	Protein of unknown function
YGR087C	***PDC6***	Pyruvate decarboxylase isozyme
YER175C	*TMT1*	Trans-aconitate Methyltransferase 1
YER065C	*ICL1*	Isocitrate lyase
YDL032W		Hypothetical ORF
YCL027W	*FUS1*	Cell-surface protein required for cell fusion
YBR147W	*RTC2*	ORF, Uncharacterized
YBR145W	***ADH5***	Alcohol dehydrogenase isoenzyme V
YPL033C	*SRL4*	Putative protein of unknown function
YPL092W	*SSU1*	Major facilitator superfamily
YOL058W	*ARG1*	Arginosuccinate synthetase
YNR068C		Putative protein of unknown function
YNR069C	***BSC5***	Bypass of Stop Codon transcript encoded by this ORF shows a high level of stop codon bypass
YNR044W	*AGA1*	a-agglutinin anchorage subunit
YNL145W	*MFA2*	a-factor mating pheromone precursor
YNL279W	*PRM1*	Pheromone-regulated multispanning membrane protein involved in membrane fusion during mating
YMR323W	*ERR3*	Protein of unknown function, has similarity to enolases

[a]Genes involved or suspected to be involved in nitrogen utilization are highlighted in *bold*.


A
WT
uga1Δ
gcn2Δ
uga3Δ
gln3Δ
gcn2Δ gln3Δ
NH4+
GABA
B
PUGA1-lacZ (fold change)
60
40
20
0
NH4+
GABA
wild-type
gcn4Δ
gln3Δ
gcn4Δ gln3Δ
uga3Δ
C
PGCRE-lacZ (nmol/min/mg)
3000
2000
1000
0
NH4+ + AA
NH4+
NH4+ + Rap
GABA
wild-type
gcn2Δ

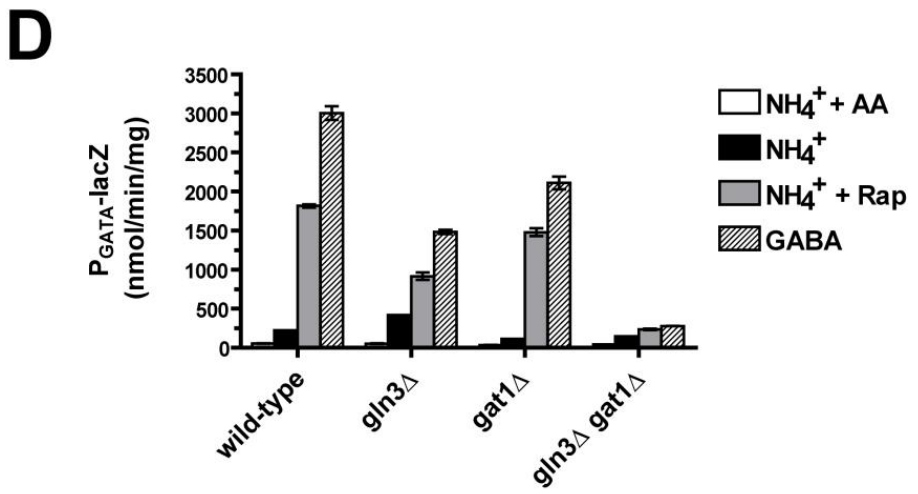


Figure 15. Gcn4p and Gln3p co-regulate gene expression in response to rapamycin treatment. (**A**) A wild-type strain, and those containing the indicated gene deletions, were grown on synthetic agar plates containing either ammonia (NH_4^+) or GABAas the nitrogen source for 3 days at 30 °C. (**B**) Yeast cells deleted for *GCN4*, *GLN3*, and *UGA3*, as indicated, were transformed with a plasmid encoding a *lacZ* reporter gene fused to the *UGA1* promoter. Cells were cultured in synthetic medium containing ammonia (NH_4^+) as the nitrogen source, switched to synthetic medium containing 10 mM GABA as the nitrogen source for 6 hours, and β-galactosidase activity was measured. The fold change in β-galactosidase activity over the cells grown in ammonia from two independent cultures ± SEM is shown. (**C**) β-galactosidase activity was measured in wild-type and *gcn2*Δ cells encoding P_{GCRE}-*lacZ*, which were grown in synthetic medium containing ammonia as the nitrogen source in the presence (NH_4^+ + AA) or absence (NH_4^+) of all twenty amino acids. Alternatively cells were treated with 200 nM rapamycin (NH_4^+ + Rap), or grown in medium containing GABA as the nitrogen source for 6 hours, as indicated. (**D**) Wild-type and mutant cells containing a P_{GATA}-*lacZ* reporter plasmid containing the consensus GATA element were grown and assayed as described in panel C.

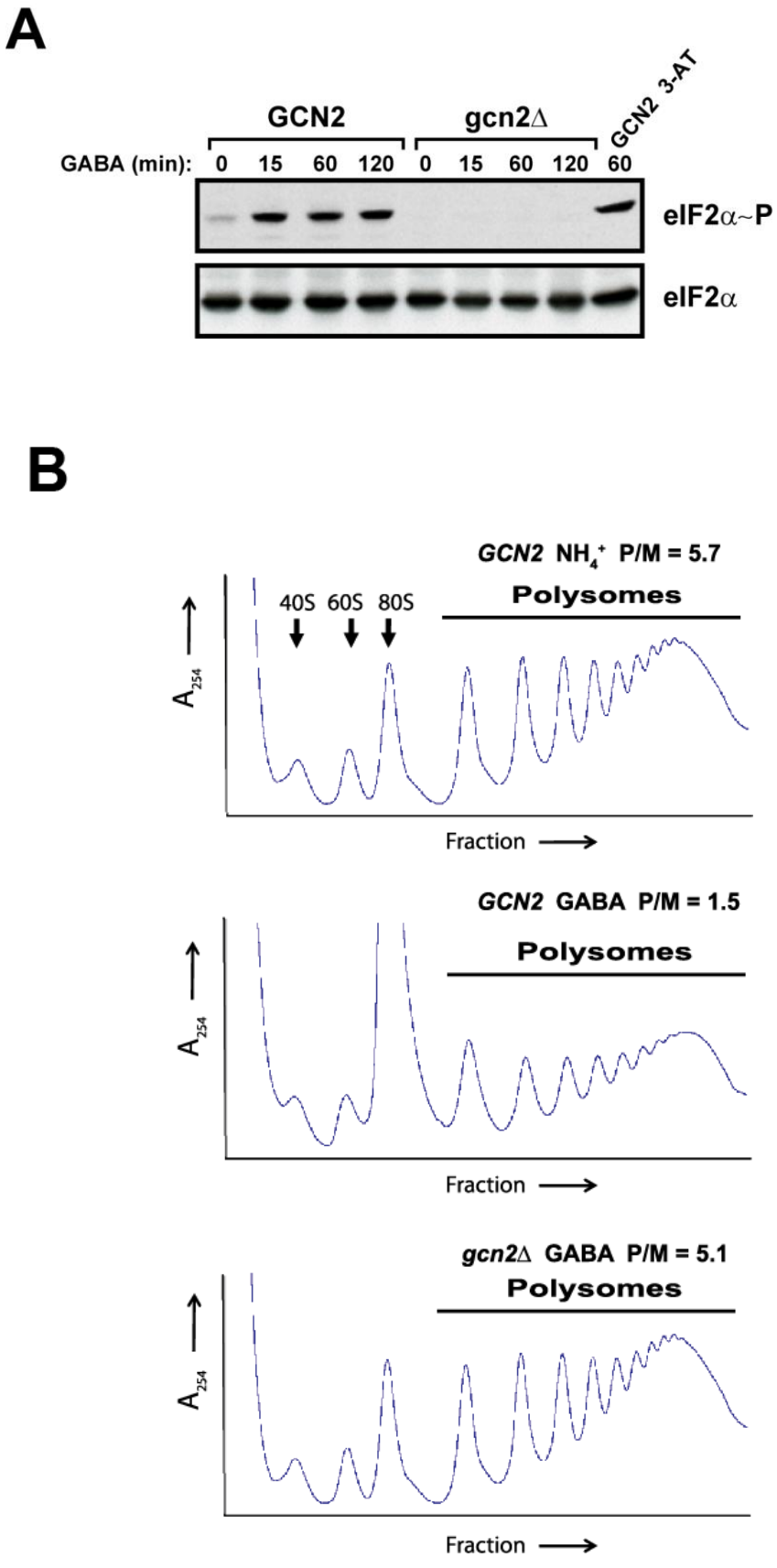
containing GABA as the nitrogen source, but had no effect on growth in media containing ammonia (Fig. 15A). By comparison, deletion of *GLN3* showed a partial reduction in growth in GABA medium, while *gcn2*Δ cells showed wild-type levels of growth. However when *gcn2*Δ and *gln3*Δ were combined, growth in GABA medium was reduced to levels comparable to cells deleted for *UGA1* or *UGA3* (Fig. 15A). These results further support the idea that the GAAC and TOR pathways function together to regulate GABA catabolism. Consistent with these findings, deletion of either *GCN2* or *GLN3* reduced *UGA1* expression as measured by a *lacZ* reporter assay in response to the addition of GABA in the medium as the sole nitrogen source (Fig. 15B). Combined *GCN2* and *GLN3* deletions led to a further lowering of P_{UGA1}-*lacZ* expression, although this reduction did not equal that found in the *uga3*Δ cells (Fig. 15B). These results suggest that the *GAAC* and TOR pathways function upstream of *UGA3* in the regulation of *UGA1* transcription in response to GABA.

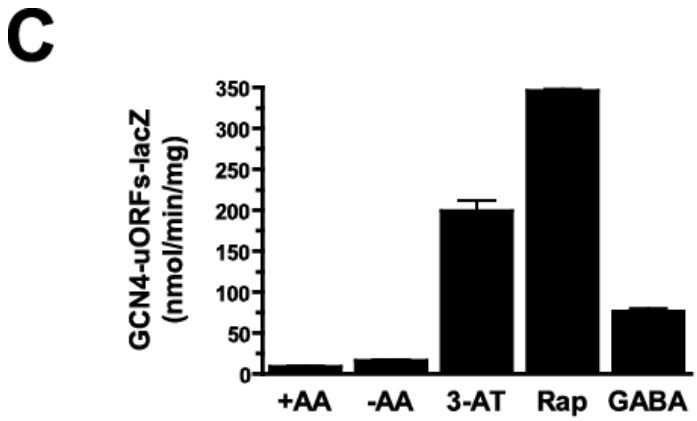
To more directly address the role of the GAAC in GABA catabolism, we assayed Gcn4p-transcriptional activation, as measured by the P_{GCRE}-*lacZ* reporter, in GABA medium. There was a marked increase in P_{GCRE}-lacZ activity in the GABA medium compared to that containing ammonia (Fig. 15C). GABA induction of Gcn4p transcriptional activity in fact exceeded that measured for rapamycin, and deletion of *GCN2* significantly reduced P_{GCRE}-*lacZ* activity in the GABA medium, as well as with rapamycin treatment. Similar assays using a lacZ reporter expressed from a promoter containing GATA elements showed that GABA was also a potent inducer of this TOR-targeted transcriptional activator, which in fact exceeded that measured following treatment with rapamycin (Fig. 15D). Deletion of *GLN3* partially reduced the P_{GATA}-lacZ

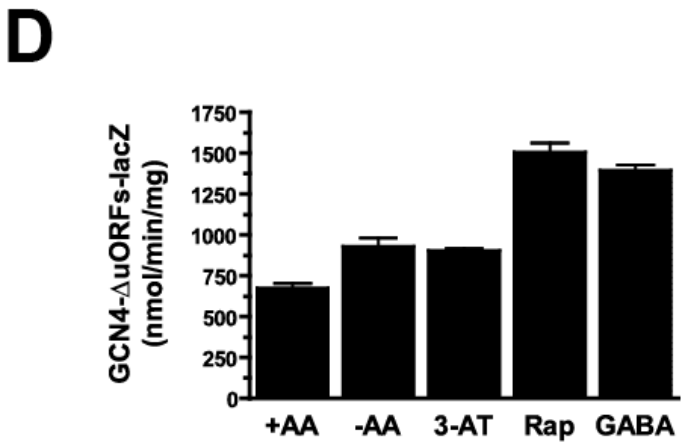
activity in response to GABA medium or in response to the addition of rapamycin to synthetic medium containing ammonia. Gat1p is another transcriptional activator in the GATA family that is induced by TOR, and *gat1*Δ cells showed a similar reduction in P_{GATA}-lacZ activity, which was further exacerbated with the combined deletion of *GLN3* and *GAT1* (Fig. 15D). It is noted that while rapamycin and GABA media significantly increased P_{GATA}-*lacZ* activity, treatment with 3-AT in fact led to a 50% reduction in P_{GATA}-*lacZ* expression (153). In our transcriptome analysis, loss of *GLN3* significantly reduced the expression of 71 genes during 3-AT stress (Table 4). This suggests that the TOR pathway per se is not repressed by 3-AT, but rather the basal activity of Gln3p is a contributor to the expression of these genes during the amino acid limiting conditions.

VII. Gcn2p phosphorylation of eIF2α is induced in cells shifted to GABA medium

We next addressed the underlying mechanisms facilitating activation of Gcn4p transcription in GABA medium. Gcn2p phosphorylation of eIF2α was induced within 15 minutes of transfer from synthetic medium supplemented with ammonia into that containing GABA (Fig. 16A). Elevated eIF2α phosphorylation was accompanied by lowered translation initiation, as judged by a sharp decrease in polysomes, coincident with elevated monosomes, as judged by sucrose gradient centrifugation (Fig. 16B). The gcn2Δ cells shifted to the GABA medium showed high levels of polysomes, supporting the idea the Gcn2 phosphorylation of eIF2α was required for this reduction in global protein synthesis. Translational expression of *GCN4*, as measured by the *GCN4-lacZ* reporter, was significantly increased in response to GABA (Fig. 16C). Consistent with


A
GCN2
gcn2Δ
GCN2 3-AT
GABA (min):
0 15 60 120 0 15 60 120 60
eIF2α~P
eIF2α
B
GCN2 NH4+ P/M = 5.7
40S 60S 80S
Polysomes
A254
Fraction
GCN2 GABA P/M = 1.5
Polysomes
A254
Fraction
gcn2Δ GABA P/M = 5.1
Polysomes
A254
Fraction


C
GCN4-uORFs-lacZ
(nmol/min/mg)
350
300
250
200
150
100
50
0
+AA
-AA
3-AT
Rap
GABA


D
GCN4-ΔuORFs-lacZ
(nmol/min/mg)
1750
1500
1250
1000
750
500
250
0
+AA
-AA
3-AT
Rap
GABA

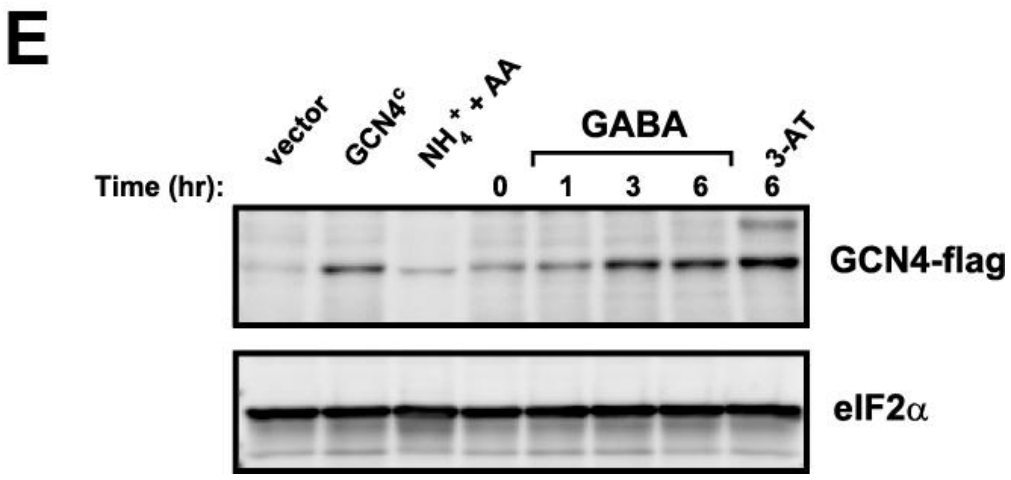

E
vector
GCN4c
NH4+ + AA
GABA
3-AT
Time (hr):
0
1
3
6
6
GCN4-flag
eIF2α

Figure 16. Gcn2p phosphorylation of eIF2α reduces global translation and enhances *GCN4* expression upon shifting to GABA medium. (**A**) Wild-type (GCN2) and *gcn2*Δ cells were grown in synthetic medium lacking amino acids and containing ammonia (NH_4^+) as the nitrogen source and then switched to minimal medium containing GABA as the nitrogen source and grown for up to 120 minutes, as indicated. As a control, *GCN2* cells were grown in ammonia containing medium supplemented with 3-AT for 60 minutes (GCN2 3-AT). Levels of phosphorylated and total eIF2α were measured by immunoblot analyses. (**B**) *GCN2* and *gcn2*Δ cells were grown in medium containing ammonia (NH_4^+) or shifted to GABA medium for 1 hour, and lysates were analyzed by sucrose gradient centrifugation. The panels show the A_{254} profile of the gradients, with free 40 S and 60 S subunits, 80 S ribosomes, and polysomes indicated. The profile for the *gcn2*Δ cells grown in synthetic medium with ammonia was not shown, as it was unchanged from the wild-type cells cultured in this medium. The ratio of polysomes (disomes or greater) compared to monosomes is illustrated above each panel. (**C)** β-galactosidase activity was measured from wild-type cells containing a *GCN4-lacZ* reporter plasmid with uORFs. Cells were grown in synthetic medium containing ammonia as the nitrogen source in the presence (+ AA) or absence (-NH_4^+) of all twenty amino acids, or were treated with 10 mM 3-AT (3-AT), 200 nM rapamycin (Rap), or grown in medium containing GABA as the nitrogen source for 6 hours, as indicated. (**D**) β-galactosidase activity was measured from wild-type cells (WY837) containing a *GCN4-lacZ* reporter plasmid without uORFs (*GCN4*-ΔuORFs-*lacZ*). Cells were grown in synthetic medium containing ammonia as the nitrogen source in the presence (+ AA) or absence (-AA) of all twenty amino acids, or were treated with 10 mM 3-AT (3-AT), 200 nM rapamycin (Rap), or grown in medium containing GABA as the only nitrogen source for 6 hours. The average β-galactosidase activity from two independent cultures is shown as the mean ± S.E. (**E**) A low-copy plasmid p1025 encoding the *GCN4* gene, with an encoded carboxy-terminal Flag epitope for detection by immunoblot, was introduced into the wild-type strain WY837. This plasmid contains the wild-type *GCN4* promoter and encoded *GCN4* uORFs. Cells were grown in synthetic medium with ammonia as the nitrogen source (0) or shifted to GABA medium for 1, 3, or 6 hours. Alternatively, these cells were cultured in SD medium containing 3-AT for 6 hours, or in SC medium containing ammonia and all amino acids (NH_4^+ + AA). For controls, the WY837 strain containing vector alone or p1025, which expresses the *GCN4* gene devoid of uORFs expressed from a constitutive ADH promoter ($GCN4^c$), were cultured in SD medium. Equal amounts of protein lysates were analyzed by immunoblot using Flag-specific antibody to visualize the tagged Gcn4p. In the *bottom* panel, eIF2α protein was measured by immunoblot to show equal amounts of total protein were analyzed in each of the lanes.

the idea that the GAAC regulates translation via uORFs in the 5-leader of the *GCN4* mRNA, high levels of *GCN4* expression were measured in cells containing a similar lacZ reporter devoid of the *GCN4* uORFs independent of the stress treatment (Fig. 16D). We also measured directly measured Gcn4p levels by immunoblot analysis and found accumulation of the Gcn4p beginning within 1 hour of shifting to GABA medium with maximum levels after 3 hours (Fig. 16E). These studies support the idea that Gcn2p phosphorylation of eIF2α triggers both global and gene-specific translational control in the GABA medium.

VIII. Sit4p facilitates *GCN4* translation in GABA medium

Gcn2p phosphorylation of eIF2α is essential for enhanced *GCN4* translational control in GABA medium as viewed by the observation that *gcn2*Δ cells and cells containing an alanine substitution at Ser-51 in eIF2α (*SUI2-S51A*) displayed significantly lowered *GCN4-lacZ* reporter activity (Figs. 17A and B). Sit4p protein phosphatase also contributes to activation of Gcn2p when TORC1 is repressed by rapamycin treatment. Consistent with an earlier report (14), the *sit4*Δ mutant suppressed *GCN4* translational control in response to rapamycin treatment (Fig. 17C). Importantly, deletion of *SIT4* also reduced *GCN4-lacZ* expressed in response to GABA medium (Fig. 17C). Together, these results are consistent with the idea that shifting to a secondary nitrogen source such as GABA relieves TOR-mediated repression of Gcn2p through a mechanism involving Sit4p protein phosphatase.

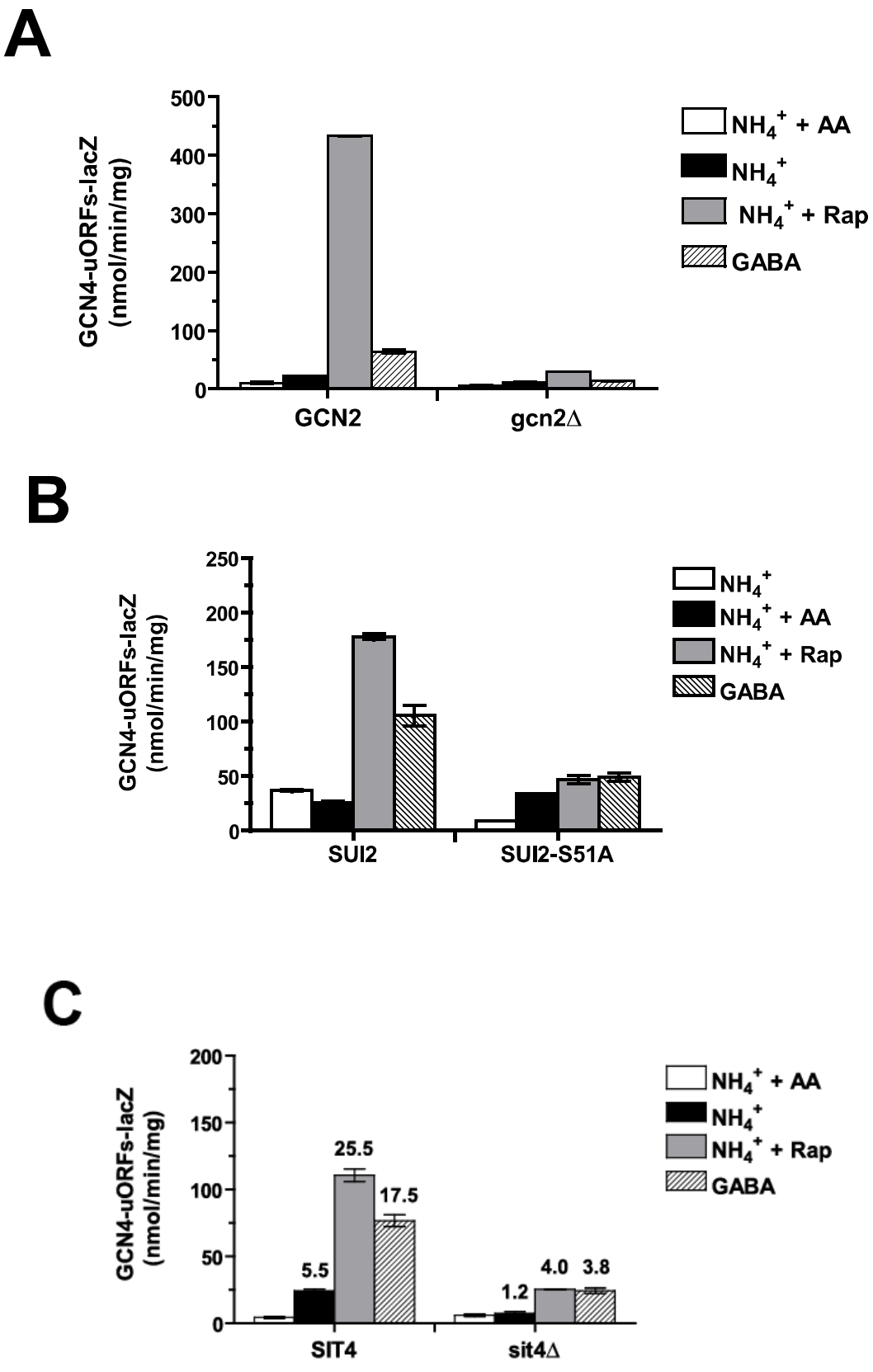
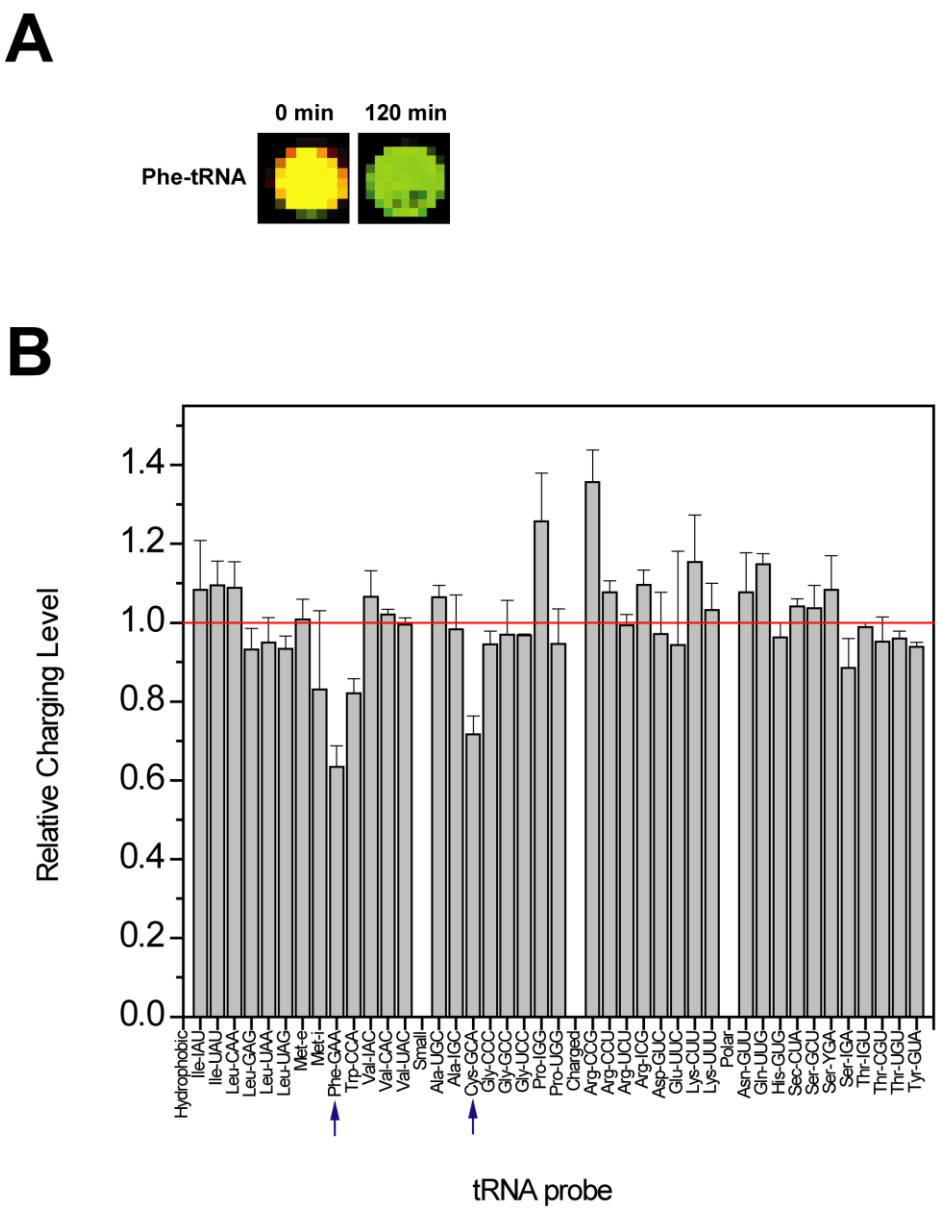

A
GCN4-uORFs-lacZ
(nmol/min/mg)
500
400
300
200
100
0
GCN2
gcn2Δ
NH4+ + AA
NH4+
NH4+ + Rap
GABA
B
GCN4-uORFs-lacZ
(nmol/min/mg)
250
200
150
100
50
0
SUI2
SUI2-S51A
NH4+
NH4+ + AA
NH4+ + Rap
GABA
C
GCN4-uORFs-lacZ
(nmol/min/mg)
200
150
100
50
0
25.5
17.5
5.5
4.0
3.8
1.2
SIT4
sit4Δ
NH4+ + AA
NH4+
NH4+ + Rap
GABA

Figure 17. Increased *GCN4* translation by the alternate nitrogen source GABA is dependent on Gcn2p and Sit4p. Wild-type (GCN2) and *gcn2*Δ **(A)**, *SUI2-SER51A* **(B)**, or *sit4*Δ **(C)** strains were cultured in synthetic medium lacking amino acids and containing ammonia (NH_4^+) as the nitrogen source and then switched to synthetic medium containing GABA as the nitrogen source and grown for 6 hours, as listed in the legend. β-galactosidase activity was measured from the *GCN2* and *gcn2*Δ cells containing a *GCN4-lacZ* reporter plasmid with uORFs (*GCN4*-uORFs-*lacZ*). The average β-galactosidase activity from three independent cultures ± S.E. is shown. In panel C, the fold-change for the different medium conditions as compared to the synthetic medium containing ammonia and all amino acids (NH_4^+ + AA) is indicated *above* each histogram.

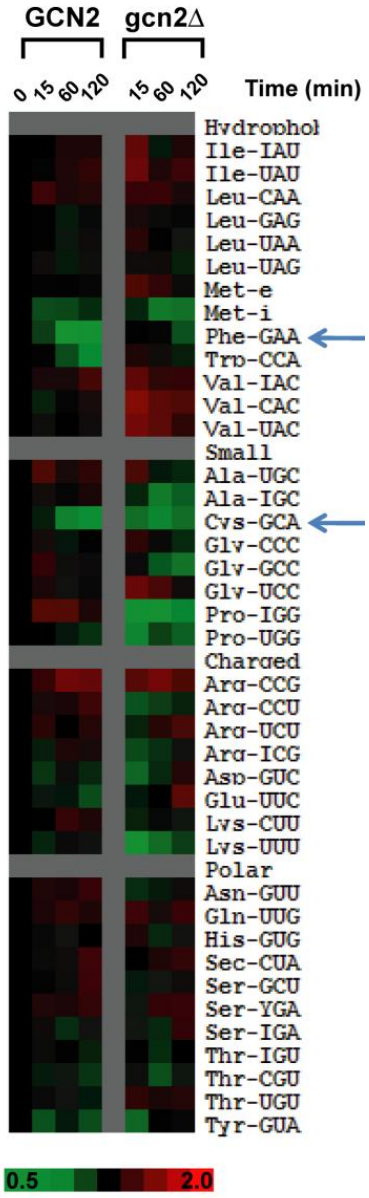
IX. Increased deacylation of $tRNA^{Asp}$ and $tRNA^{Phe}$ in cells shifted to GABA medium

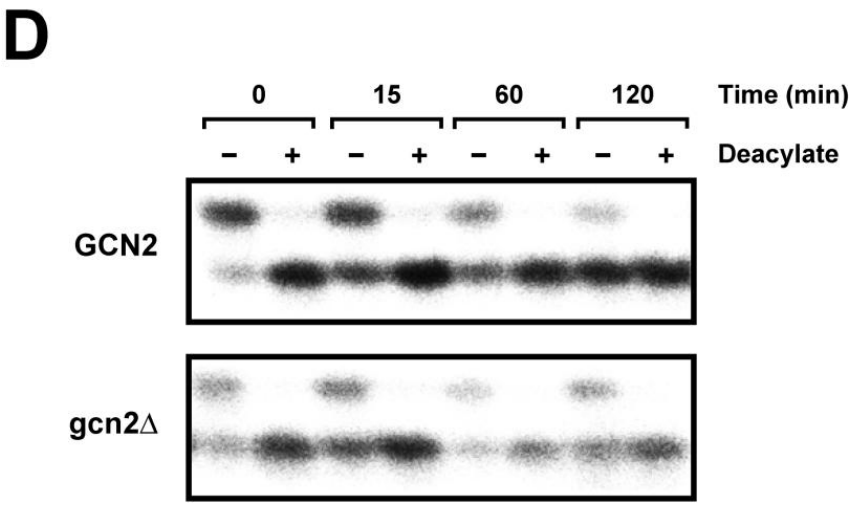
Our studies suggest that repression of TORC1 contributes to activation of the GAAC in response to a shift from synthetic medium supplemented with ammonia into that containing GABA. Elevated uncharged tRNA levels are also central to activation of Gcn2p. To directly address whether the levels of tRNA charging are reduced upon a shift to GABA medium, we measured changes in charging of all tRNA species by a method involving tRNA microarrays that includes complementary probes to each chromosomal-encoded tRNA (22). A prototrophic strain, and its *gcn2*Δ counterpart, were shifted from SD medium to synthetic medium containing GABA for up to 120 minutes, and RNA was extracted under mild acidic conditions which retain aminoacylated tRNAs. The RNA sample was split into two parts. One half was subjected to periodate oxidation, destroying the 3'-ends of all uncharged tRNAs, while the other half served as a control and was not treated with periodate. Both samples were then deacylated using alkaline pH and a fluorescent-tagged oligonucleotide was ligated onto only those tRNAs with intact 3'-ends. Both samples were labeled with Cy3 or Cy5 fluorophore. After fluorescent labeling, tRNA preparations with opposite fluorophores, for example charged tRNA with Cy5 and total tRNA with Cy3, were combined and hybridized to microarrays.

The array results presented as histograms and heat maps showed the relative tRNA charging levels between cells shifted to GABA medium compared to the SD control (Fig 18). Within 60 minutes of the shift of the wild-type cells to GABA medium, there was a significant reduction in the charging of $tRNA^{Phe}$ and $tRNA^{Cys}$, along with a more modest reduction in $tRNA^{Trp}$ (Figs. 18A-C). These tRNA charging levels were


A
0 min
120 min
Phe-tRNA
B
Relative Charging Level
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Hydrophobic
Ile-IAU
Ile-UAU
Leu-CAA
Leu-GAG
Leu-UAA
Leu-UAG
Met-e
Met-i
Phe-GAA
Trp-CCA
Val-IAC
Val-CAC
Val-UAC
Small
Ala-UGC
Ala-IGC
Cys-GCA
Gly-CCC
Gly-GCC
Gly-UCC
Pro-IGG
Pro-UGG
Charged
Arg-CCG
Arg-CCU
Arg-UCU
Arg-ICG
Asp-GUC
Glu-UUC
Lys-CUU
Lys-UUU
Polar
Asn-GUU
Gln-UUG
His-GUG
Sec-CUA
Ser-GCU
Ser-YGA
Ser-IGA
Thr-IGU
Thr-CGU
Thr-UGU
Tyr-GUA
tRNA probe

C


GCN2
gcn2Δ
0 15 60 120
15 60 120
Time (min)
Hydrophol
Ile-IAU
Ile-UAU
Leu-CAA
Leu-GAG
Leu-UAA
Leu-UAG
Met-e
Met-i
Phe-GAA
Trp-CCA
Val-IAC
Val-CAC
Val-UAC
Small
Ala-UGC
Ala-IGC
Cys-GCA
Gly-CCC
Gly-GCC
Gly-UCC
Pro-IGG
Pro-UGG
Charged
Arg-CCG
Arg-CCU
Arg-UCU
Arg-ICG
Asp-GUC
Glu-UUC
Lys-CUU
Lys-UUU
Polar
Asn-GUU
Gln-UUG
His-GUG
Sec-CUA
Ser-GCU
Ser-YGA
Ser-IGA
Thr-IGU
Thr-CGU
Thr-UGU
Tyr-GUA
0.5
2.0


D
0
15
60
120
Time (min)
– + – + – + – +
Deacylate
GCN2
gcn2Δ

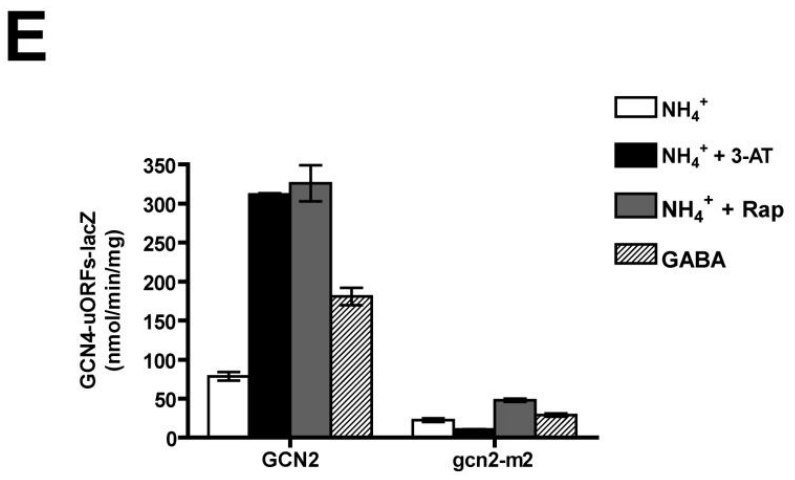

E
NH4+
NH4+ + 3-AT
NH4+ + Rap
GABA
GCN4-uORFs-lacZ (nmol/min/mg)
350
300
250
200
150
100
50
0
GCN2
gcn2-m2

Figure 18. Increased uncharged tRNA levels in cells shifted to GABA medium. The protrophic strain WY798 and isogenic *gcn2*Δ counterpart (WY799) were cultured in SD medium and then shifted to synthetic medium containing GABA as the sole nitrogen source for 15, 30, and 120 minutes. The charging of tRNA genome-wide was measured by the microarray method. (**A**) Scanned flourescent images of $tRNA^{Phe}$ hybridized to the complementary probe in the microarrays. The tRNA preparations were cultured in SD medium (*0 min*) or GABA medium for 120 minutes (120 min). *Yellow* represents no change in the charging of $tRNA^{Phe}$ and green indicates low tRNA charging. (**B**) The relative levels of tRNA charging are presented as the ratio of each charged tRNA prepared from the wild-type strain cultured in GABA medium for 60 minutes compared to those cultured in SD medium. The *x* axis lists each of the different tRNAs, collated into hydrophobic, small, charged and polar groups. The value of 1.0 in the *y* axis indicates that the tRNA charging in cells cultured in GABA is equal to that in the SD control. Values less than 1.0 indicate reduced tRNA charging, while values greater than 1.0 denote tRNA charging that is greater upon shift of the cells to GABA medium. Error bars represent ± S.E. (**C**) Heat map representations of genome-wide tRNA charging in response a shift to GABA medium. The levels of tRNA charging were measured in the *GCN2* and *gcn2*Δ strains upon shifting to GABA medium for 15, 60, and 120 minutes. Those cells that were shifted to SD medium as a control are represented as 0. Green indicates decreased tRNA charging in the GABA medium compared to the SD control, while red represents enhanced tRNA charging, as listed on the scale to the right of the figures. (**D**) Northern blot analysis of acid-denaturing gels measuring the charging of $tRNA^{Phe}$ in the *GCN2* and *gcn2*Δ strains upon shifting the cultures from SD medium to GABA medium for up to 120 minutes, as indicated. The panels include an autoradiogram representing hybridization of a radiolabeled probe complementary to charged (slower migrating band) and uncharged (faster migrating band) $tRNA^{Phe}$. As a control, the $tRNA^{Phe}$ was deacylated prior to the Northern analysis (+) and compared to samples that were not subjected to deacylation *in vitro* prior to the Northern analysis (-). (**E**) Strain RY139 (*gcn2*Δ containing plasmid pYB41 encoding a *GCN4-lacZ* reporter with uORFs intact was transformed with a low-copy plasmid encoding *GCN2*, mutant *gcn2-m2*, or vector alone. Cells grown in synthetic medium containing ammonia as the nitrogen source (NH_4^+) were treated with 10 mM 3-AT (NH_4^+ + 3-AT) or 200 nM rapamycin (NH_4^+ + Rap), or grown in medium containing GABA (GABA) as the nitrogen source for 6 hours. The average β-galactosidase activity measured from three independent cultures ± S.E. is shown.

further diminished with culturing in this medium for 120 minutes (Fig. 18C). The *gcn2*Δ strain showed a different pattern of charging genome-wide, with increased deacylation of $tRNA^{Cys}$, $tRNA^{Lys}$, $tRNA^{Pro}$, in both the SD and synthetic GABA medium (Fig 18C). Upon shifting to the GABA medium there were additional reductions in tRNA charging levels, including that of the initiator $tRNA_i^{Met}$.

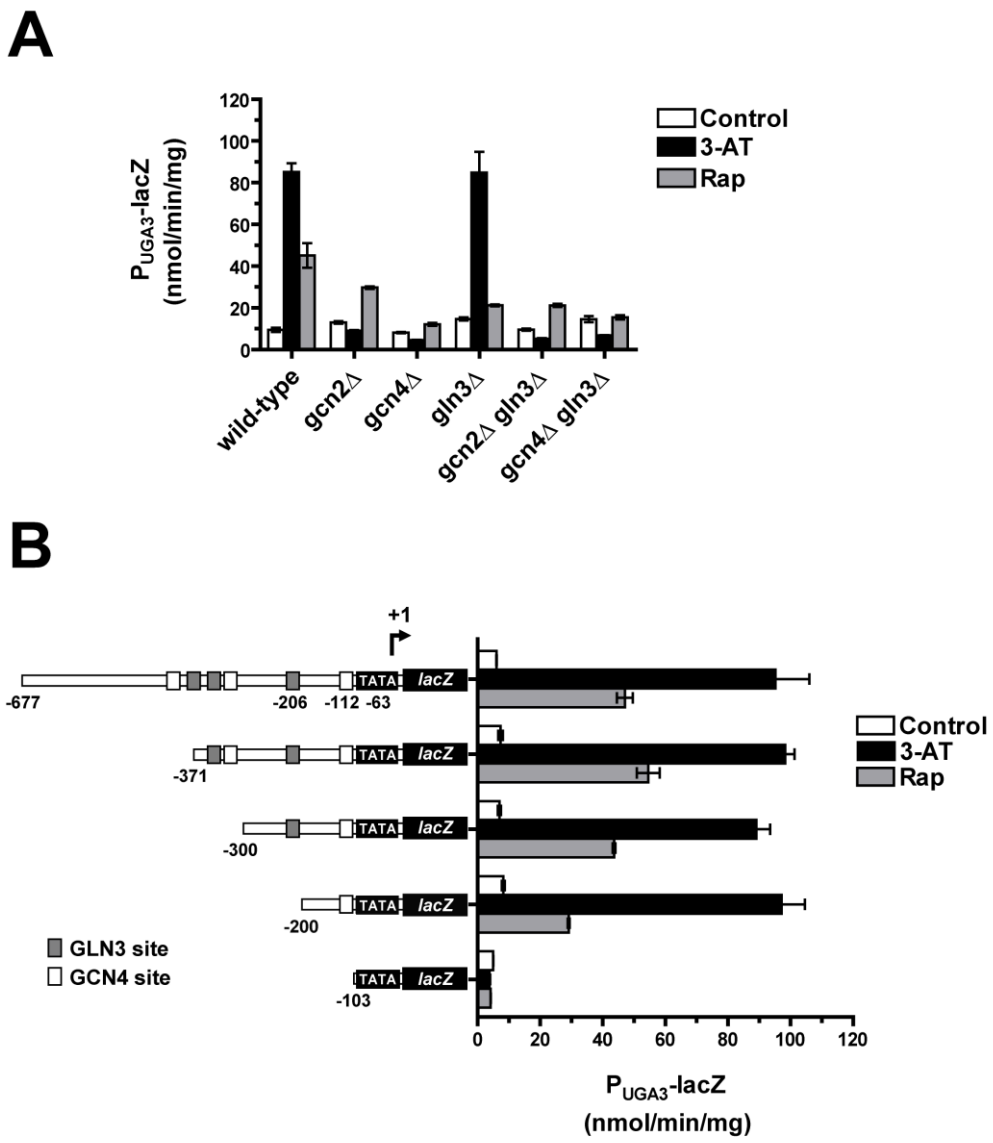
The reduced charging of $tRNA^{Phe}$ in the wild-type cells was confirmed by Northern analysis (Fig. 18D). The tRNA preparations subjected to deacylation *in vitro* showed uniformly uncharged $tRNA^{Phe}$, presented as the faster migrating band. The $tRNA^{Phe}$ was charged at >75% in SD medium, with progressive reductions in charging levels upon shifting to the GABA medium, and with the greatest degree of deacylation at 120 minutes. The charging levels of $tRNA^{Phe}$ were retained in the *gcn2*Δ cells cultured in SD and the synthetic GABA medium.

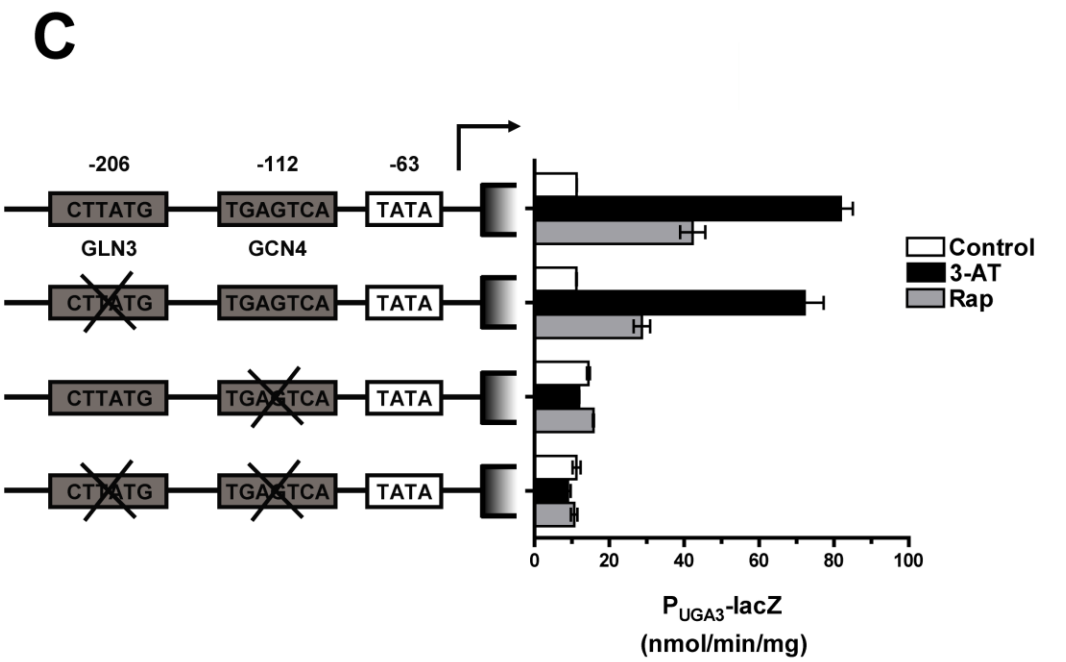
The enhanced levels of uncharged tRNA suggests that induced Gcn2p phosphorylation of eIF2α upon shifting to the GABA medium involves Gcn2p binding to uncharged tRNA, along with TORC1 signaling. To further address the role of activation of Gcn2p by uncharged tRNA in response to the shift to the GABA medium *in vivo*, we measured induction of *GCN4* translational control in cells containing a mutant version of *GCN2* (*gcn2-m2*) that contains missense mutations previously shown to block binding to uncharged tRNA. We reasoned that if uncharged tRNA is central for activation of Gcn2p in response to a shift to synthetic GABA medium, that mutations that blocked Gcn2p binding to uncharged tRNA would thwart activation of the GAAC. Indeed we found that *gcn2-m2* mutation blocked enhanced *GCN4-lacZ* expressed in response to GABA medium (Fig. 18E). These findings suggest that enhanced levels of uncharged tRNA

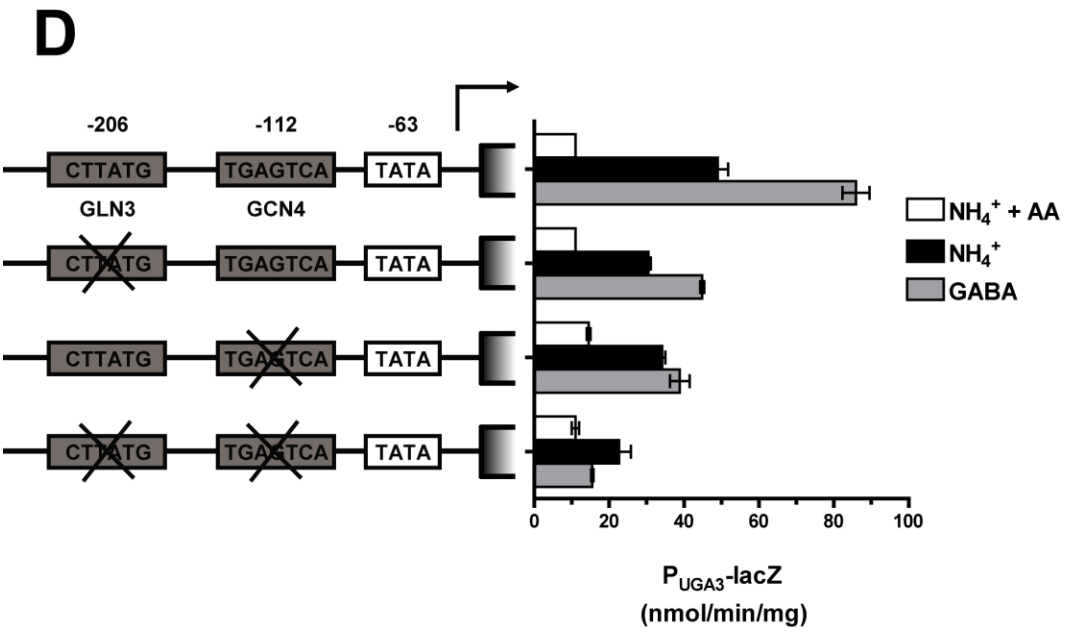
contribute to activation of Gcn2p and the GAAC upon shifting from SD to synthetic GABA medium.

X. Gcn4p and Gln3p activate *UGA3* transcription

Our transcriptome studies suggested that Gcn4p and Gln3p activate *UGA3* transcription in response to stress, and therefore the role of the GAAC and TOR pathways is to coordinate the transcription of *UGA1*, and other GABA catabolic genes. This idea is further supported by our observation that expression of a *lacZ* reporter fused to the *UGA3* promoter (P_{UGA3}) was increased in response to rapamycin or 3-AT treatment (Fig. 19A). Deletion of either *GCN2* or *GCN4* significantly lowered *UGA3* transcription in response to either stress. By comparison, loss of *GLN3* lowered P_{UGA3}-*lacZ* expression only during rapamycin exposure, with no change in the 3-AT medium (Fig. 19A). There are four predicted Gln3p binding sites in the P_{UGA3} region, and two Gcn4p binding elements (Fig. 19B). To determine the minimum elements in the P_{UGA3} required for enhanced transcription in response to 3-AT or rapamycin stress, a processive 5'-deletion analysis was carried out in the P_{UGA3}-*lacZ* reporter. Removal of the most 5'-elements, including two Gln3p-binding sites and a single Gcn4p-binding element, in the $P_{UGA3\text{-}300}$-*lacZ* reporter construct did not significantly reduce transcription in response to either 3-AT or rapamycin treatment (Fig. 19B). Further deletion of the Gln3p binding site in the P_{UGA3} lowered β-galactosidase activity by 2-fold during rapamycin treatment, but had no effect in response to 3-AT. Importantly, removal of the lone GCRE blocked *UGA3* transcription in response to either stress condition, suggesting that Gcn4p binding to the P_{UGA3} region is central for increased *UGA3* transcription (Fig. 19B).


A
120
100
80
60
40
20
0
P UGA3 -lacZ
(nmol/min/mg)
Control
3-AT
Rap
wild-type
gcn2Δ
gcn4Δ
gln3Δ
gcn2Δ gln3Δ
gcn4Δ gln3Δ
B
+1
TATA
lacZ
-677
-206
-112
-63
-371
-300
-200
-103
GLN3 site
GCN4 site
Control
3-AT
Rap
0
20
40
60
80
100
120
P UGA3 -lacZ
(nmol/min/mg)


C
-206
-112
-63
CTTATG
TGAGTCA
TATA
GLN3
GCN4
Control
3-AT
Rap
0
20
40
60
80
100
P UGA3 -lacZ
(nmol/min/mg)


D
-206
-112
-63
CTTATG
TGAGTCA
TATA
GLN3
GCN4
NH4+ + AA
NH4+
GABA
0
20
40
60
80
100
P UGA3 -lacZ
(nmol/min/mg)

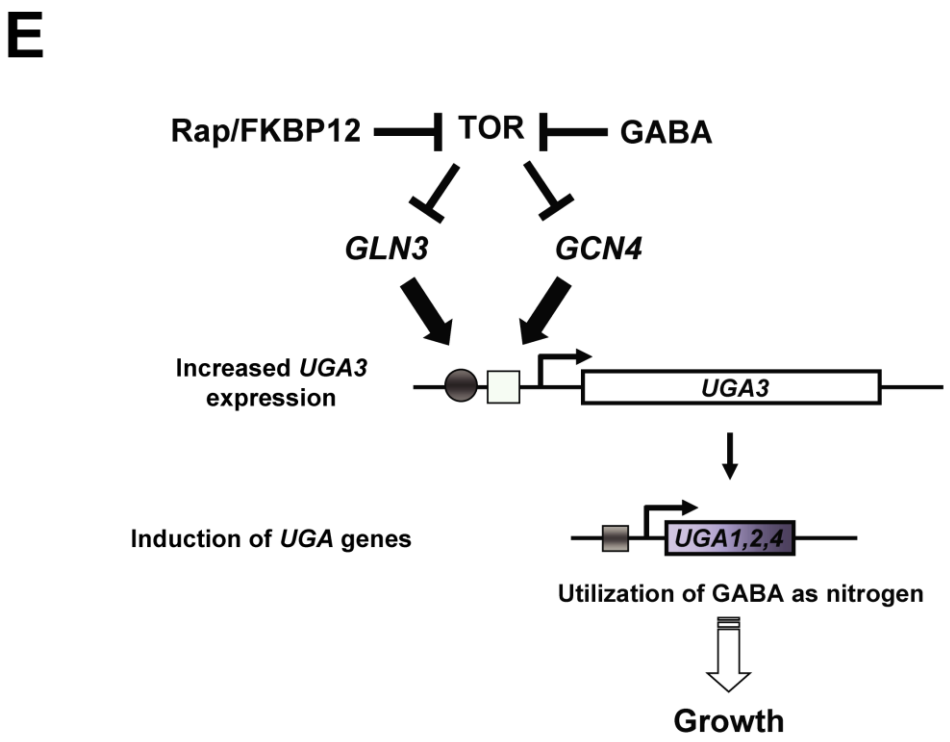


Figure 19. Gcn4p and Gln3p co-regulate the *UGA3* promoter. (**A**) A P_{UGA3}-*lacZ* reporter plasmid was introduced into wild-type cells, or cells deleted for *GCN2*, *GCN4*, and *GLN3*, individually or in combination, as indicated. Cells were cultured in synthetic complete medium supplemented with all amino acids except histidine, treated with 3-AT, rapamycin (Rap), or no treatment (Control) for 6 hours, and β-galactosidase activity was measured. The average β-galactosidase activity from two independent cultures ± S.E. is shown. (**B**) Wild-type cells containing a *lacZ* reporter gene under the control of the *UGA3* promoter (-677) or deletions of the *UGA3* promoter (-371, -300, -200, or -103), as indicated, were cultured and treated as in panel A. Predicted Gln3p and Gcn4p binding sites in the *UGA3* promoter are indicated. (**C**) The consensus Gln3p (GATA) and Gcn4p (GCRE) binding sites present in the minimal *UGA3* promoter at -206 and -112, respectively, were mutated by site-directed mutagenesis, as indicated. Wild-type cells containing a *lacZ* reporter gene under the control of the wild-type minimal *UGA3* promoter (-300), a minimal promoter containing a mutant Gln3p (GATA) and Gcn4p (GCRE) binding sites, individually or in combination, as indicated were cultured and treated as described in panel A. (**D**) Wild-type cells containing P_{UGA3}-*lacZ* reporter genes as described in C were cultured in synthetic medium containing ammonia as the nitrogen source in the presence (NH_4^+ + AA) of all twenty amino acids, absence (NH_4^+) of all twenty amino acids, or grown in medium containing GABA as the nitrogen source as for 6 hours, as indicated. (**E**) Model depicting the role TOR-mediated regulation of Gln3p and Gcn4p activity in regulation of *UGA3* mRNA expression and subsequent utilization of GABA as a nitrogen source in yeast.

To further address the roles of the Gcn4p and Gln3p-binding elements in *UGA3* transcription, these elements were mutated individually or in combination in the proximal -300 version of the of the P_{UGA3}-*lacZ* reporter, which retained maximal transcription in response to either 3-AT or rapamycin (Fig. 19C). Consistent with our deletion analysis, mutations in the Gcn4p binding element blocked $P_{UGA3\text{-}300}$-*lacZ* expression in response to either stress condition. By comparison, alteration of the Gln3p binding element partially lowered $P_{UGA3\text{-}300}$-lacZ activity in response to rapamycin.

We also addressed whether these two binding elements are important for *UGA3* transcription when GABA is the sole nitrogen source in the medium. The $P_{UGA3\text{-}300}$-*lacZ* reporter were assayed in cells cultured in medium containing ammonia and a complete complement of amino acids, or medium devoid of amino acids and only GABA as the nitrogen source. GABA medium led to an 8-fold increase in $P_{UGA3\text{-}300}$-lacZ activity (Fig. 19D). Mutations in either the Gcn4p or the Gln3p binding element significantly reduced $P_{UGA3\text{-}300}$-*lacZ* expression in GABA medium. Therefore, Gcn4p and Gln3p are central for increasing *UGA3* transcription in response to a variety of nutrient stresses. These findings support the idea that Gcn4p is a major transcriptional effector of the TORC1 pathway, and that this regulatory pathway has an important role in the assimilation of secondary nitrogen sources, such as GABA (Fig. 19E).

DISCUSSION

I. Central questions addressed in this microarray study

This study addressed the regulatory linkages between the GAAC and TOR pathways in directing the transcriptome in response to amino acid starvation and rapamycin treatment. Three central questions were addressed. The first central question is whether the transcriptome directed by Gcn4p in response to amino acid depletion is similar to that in rapamycin treatment, or can different stress conditions allow for significant variations in the Gcn4p-directed transcription? We found that there is a core of 57 genes that are activated by Gcn4p in response to either stress condition; these included genes involved in amino acid biosynthesis, nitrogen utilization, signaling, and gene expression (Fig. 10C). Among these core genes, we focused on the relationship between the GAAC and nitrogen utilization. Specifically, we delineated the underlying mechanisms by which Gcn4p facilitates GABA utilization and the processes contributing to the activation of the GAAC upon shifting to this secondary nitrogen source. These results indicate that the GAAC has a focal set of genes that are central for cellular adaptation to different stresses. Gcn4p and the GAAC also retain versatility to direct stress-specific gene expression, as illustrated by the large collection of genes induced specifically by 3-AT or rapamycin treatment (Fig. 10A). The Gcn4p-targeted genes activated specifically by histidine depletion emphasized amino acid and intermediary metabolism, while those induced by rapamycin included generation and utilization of energy-related compounds (supplemental Tables S2 and S3, (153)).

The second central question concerned the role of Gcn4p in TOR-directed gene expression and the mechanisms underlying the regulatory linkages between the GAAC

and TOR pathways. Importantly, we found that Gcn4p is a major transcriptional effector of the TOR pathway, with the number of genes requiring Gcn4p for activation in response to rapamycin treatment being similar to that dependent on the canonical Gln3p transcription factor (Table 4). In response to rapamycin, Gcn4p induced genes involved in amino acid biosynthesis, intermediary metabolism, and transport processes. Furthermore, analogous to the TOR pathway, Gcn4p targets many genes that are central for nitrogen utilization.

The GAAC and TOR pathways intersect at multiple points to regulate the transcriptome. Rapamycin inhibition of TORC1 is suggested to reduce inhibitory phosphorylation of Gcn2p by activating type 2A-related protein phosphatases, such as Sit4p, and by inhibiting an unknown protein kinase (14). Our study suggests that the shift to a secondary nitrogen sources, such as GABA, is a physiological condition that releases TORC1 inhibition of Gcn2p and the GAAC. We propose that there is a dual mechanism regulating the GAAC upon shifting to GABA medium. Gcn2p activation can occur by both the release of TOR repression of Gcn2p and activation of this eIF2α kinase by uncharged tRNAs that accumulate in response to a shift to a secondary nitrogen source. We found that a shift to GABA medium led to significant deacylation of tRNAs ($tRNA^{Phe}$ and $tRNA^{Cys}$) (Fig. 18), which is suggested to also contribute to activation of Gcn2p by direct binding to this eIF2α kinase. It is noted that deletion of *GCN2* alters the pattern of accumulated uncharged tRNA (Fig. 18C). In the *gcn2*Δ cells grown in synthetic medium containing ammonia there were significant levels of many different uncharged tRNAs, including $tRNA^{Asp}$, $tRNA^{Cys}$, $tRNA^{Lys}$, $tRNA^{Pro}$ and $tRNA^{Tyr}$. This finding suggests that loss of Gcn2p creates a deficiency in the amino acid biosynthetic

pathways that can lead to significant levels of different deacylated tRNAs. Interestingly, upon shifting the *GCN2*-deficient cells to GABA, there continued to be significant levels of uncharged tRNA, albeit the pattern was modified compared to the wild-type strain (Fig. 18C).

Additional points of intersection occur downstream in the TOR and GAAC pathways. The Gcn4p activation core includes *GLN3*, suggesting that activation of the GAAC can increase Gln3p activity at least under some nutrient conditions (Fig. 10C). Therefore, the requirement for Gcn4p for stress-induced expression of some genes may in indirect. The transcriptional activators Gcn4p and Gln3p also can coordinately induce transcription of target genes, such as *UGA3,* by binding at different elements within their respective promoters (Fig. 19E).

The third central question is whether Gcn2p can regulate the transcriptome independent of Gcn4p or vice versa? The predominant picture is that genes requiring Gcn2p for induction in response to either rapamycin or 3-AT were also dependent on Gcn4p. These results support the model in which the primary role of Gcn2p in yeast is to enhance Gcn4p expression in response to stress. Included among those genes requiring Gcn2p for activation in response to both 3-AT and rapamycin are *ARO9* and *ARO10* (Figs. 13A and B). Both genes are central for catabolism of aromatic amino acids, and interestingly their induced mRNA expression was independent of *GCN4*. However, deletion of either *GCN2* or *GCN4* led to a significant growth defect in medium containing phenylalanine as the sole nitrogen source when combined with *aro80*Δ (Fig. 13D). These results suggest that although Gcn4p is not directly involved in *ARO9* and *ARO10*

transcription, Gcn4p contributes to the metabolic conditions required for assimilation of aromatic amino acids.

It is also noteworthy that almost a third of the genes targeted by Gcn4p displayed significantly reduced requirements for Gcn2p, suggesting that basal levels of Gcn4p retain important biological functions (Table 4). This idea is consistent with the findings that the *gcn4*Δ cells have a growth defect in the absence of supplemented amino acids, while loss of *GCN2* in an otherwise prototrophic strain does not have a phenotype in SD medium devoid of amino acids (Fig. 13D). Analogous to Gcn4p, the basal activity of Gln3p is also suggested to have activating functions in the transcriptome. This was illustrated in cells treated with 3-AT, where 71 genes required Gln3p for full induction (Table 4). However, 3-AT in fact did not lead to any increase in the expression of the P_{GATA}-*lacZ* reporter (153). This suggests that the basal activity of Gln3p is a significant contributor to the transcriptome during amino acid starvation, although the precise mechanisms are currently unclear. Supporting this idea is the finding that deletion of this transcription factor reduces growth in 3-AT containing medium (Fig. 8A).

II. Gcn4p directs different transcriptome programs in response to diverse stresses

Our microarray analysis identified a collection of genes, designated the Gcn4p activation core (GAC), which are transcriptionally induced during either amino acid starvation or rapamycin treatment. Gcn4p can also increase the expression of additional genes that are unique to the specific stress (Table 4). Currently, we understand only general mechanistic schemes for how Gcn4p can delineate between core and stress-

specific gene transcription. Gcn4p activation of transcription is largely thought to be a consequence of its increased concentration in the cell, which involves translational control induced by eIF2α phosphorylation and its turnover, triggered by Pho85p/Pcl5p (73,171,177). Elevated levels of Gcn4p lead to its enhanced binding at the GCREs in the promoters of its target genes, which then serves to recruit different co-activators that facilitate association with RNA polymerase II (178).

For stress-specific gene transcription, one anticipates additional mechanisms for controlling Gcn4p activity, or supplementary transcription factors that interact with the promoters of a subset of Gcn4p-targeted genes and are regulated by alternative stress pathways. Supporting the idea that additional mechanisms contribute to Gcn4p activity, we found that GABA induction of Gcn4p transcriptional activity in fact exceeded that measured for other stresses, 3-AT and rapamycin, despite there being a more moderate inducer of *GCN4* translational control (Figs. 15C and 16B). Regarding supplemental transcription factors, we showed that Gcn4p and Gln3p were required for full induction of 24 different genes during rapamycin stress, including regulation of the *UGA3* promoter in response to rapamycin treatment, as well as during a shift to GABA medium (Table 5 and Figs. 19C and 19D). Cooperation between Gcn4p and a second transcription factor, which can be regulated independent of the Gcn4p and the GAAC during certain stresses, would facilitate variations in the transcriptome. Many of these central concepts concerning Gcn4p and stress-specific gene expression are germane to the transcriptome induced by eIF2α phosphorylation in mammalian cells subjected to different stress arrangements, such as those influenced by nutrient deficiencies or unfolded proteins (10,179-181).

Gcn4p was also shown to be required for repression of a number of genes in response to 3-AT or rapamycin, with 16 genes constituting the so-called Gcn4p repression core (Fig. 10D and supplemental Table S5, (153)). The mechanism by which Gcn4p contributes to repressed transcription is not understood. It has been proposed that elevated levels of Gcn4p may squelch the transcription of certain target genes by binding to and impeding the function of key co-activator proteins, therefore compromising the induction of these target genes (82,182). Interestingly, many of these Gcn4p repression core genes have GCREs in proximity to their promoters, which suggests that Gcn4p repression may result through direct DNA binding. Gln3p contributed to ≥2-fold repression of an even greater number of genes compared with Gcn4p (396 *versus* 197 genes, Table 4 and supplemental Table S2 (153)). The underlying mechanisms for Gln3p repression are also not clear. It has been suggested that Gln3p repression of transcription of amino acid biosynthetic genes may be indirect through imbalanced changes in the expression of permease genes that facilitate nutrient uptake (183). We note that many of the genes requiring *GLN3* for repression have identifiable GATA elements in their promoters (supplemental Table S5, (153)), suggesting that Gln3p may repress selected promoters by direct binding.

III. TOR regulates the GAAC to facilitate utilization of secondary nitrogen sources

Our study suggests that TOR regulation of the GAAC is central for adaptation to secondary nitrogen sources in the medium. Introduction of GABA as a nitrogen source enhances Gcn2p phosphorylation of eIF2α, leading to increased Gcn4p translation and

transcriptional activity (Figs. 15C, 16A, C, and D). Supporting the idea that GABA activation of Gcn2p is facilitated through inhibition of TORC1 is the finding that GABA induces Gln3p activity (Fig. 15D), and that GABA induction of *GCN4* mRNA translation is diminished by loss of Sit4p (Fig. 17C), which is similar to that reported for rapamycin treatment (14). By contrast, amino acid starvation elicited by 3-AT does not lead to repression of TORC1, as judged by only diminished Gln3p-directed transcription during this stress condition, and activation of Gcn2p in response to 3-AT does not require Sit4p (14).

Together, these results support a model in which dual mechanisms contribute to activation of Gcn2p and the GAAC in response to changes in nutrient availability. In the first mechanism, amino acid deprivation, such as that elicited by 3-AT, increases the levels of uncharged tRNA. Elevated levels of uncharged tRNA would then directly bind to the HisRS-related domain of Gcn2p, resulting in enhanced Gcn2p phosphorylation of eIF2α (Fig. 20). The second mechanism would occur when yeast cells are shifted to medium containing certain secondary nitrogen sources, such as GABA. Repression of TORC1 upon shifting to GABA medium can induce eIF2α kinase activity by a mechanism consistent with release of inhibitory phosphorylation of Gcn2p (Fig. 20). This release of Gcn2p inhibitory phosphorylation would involve the signaling pathway including *TAP42* and the type 2A protein phosphatases encoded by *SIT4* and the *SIT4*-associated protein (*SAP*) genes.

Uncharged tRNA is also suggested to be an activating ligand for Gcn2p in GABA medium, therefore TORC1 signaling and Gcn2p binding to uncharged tRNA appear to work in combination to enhance Gcn2p phosphorylation of eIF2α in response to a shift to

secondary nitrogen sources. It is noteworthy that phosphorylation of eIF2α occurs within 15 minutes of a shift from SD to synthetic GABA medium and is retained after 120 minutes (Fig. 16A). However, significant deacylation of $tRNA^{Phe}$ and $tRNA^{Cys}$ occurs after culturing for 1 hour in the GABA medium (Fig. 18C). The timing of these events suggests that the two mechanisms may contribute at different periods during the course of activation of Gcn2p. A central feature in this mechanism of activation is that the TORC1 and Gcn2p protein kinases are reciprocally regulated in response to changes in nitrogen source, which would contribute to their opposing control of protein synthesis and Gcn4p activation (Fig. 20).

Godard and colleagues have also suggested a role for Gcn4p and the GAAC in nitrogen catabolism (159). In this earlier study, transcriptome measurements were carried out in the yeast Σ1278b strain grown in minimal medium containing one of 21 different nitrogen sources. The majority of these nitrogen sources were classified into two different groups, an A group of nitrogen compounds that supported rapid growth, and a B group of nitrogen sources that supported only slow growth. Growth in the group B nitrogen compounds, including many different amino acids (leucine, isoleucine, methionine, threonine, tryptophan, and tyrosine) led to increased expression of many genes linked with Gcn4p. These results suggested that the GAAC is activated in yeast cultured in medium with poorer nitrogen sources. In fact many of the genes induced during culturing in media with group B nitrogen compounds were included in the Gcn4p activation core, as listed in the genes highlighted in blue text in Fig. 10C (supplemental Table S3 (153)). In addition to GABA catabolism, we found that the GAAC facilitates

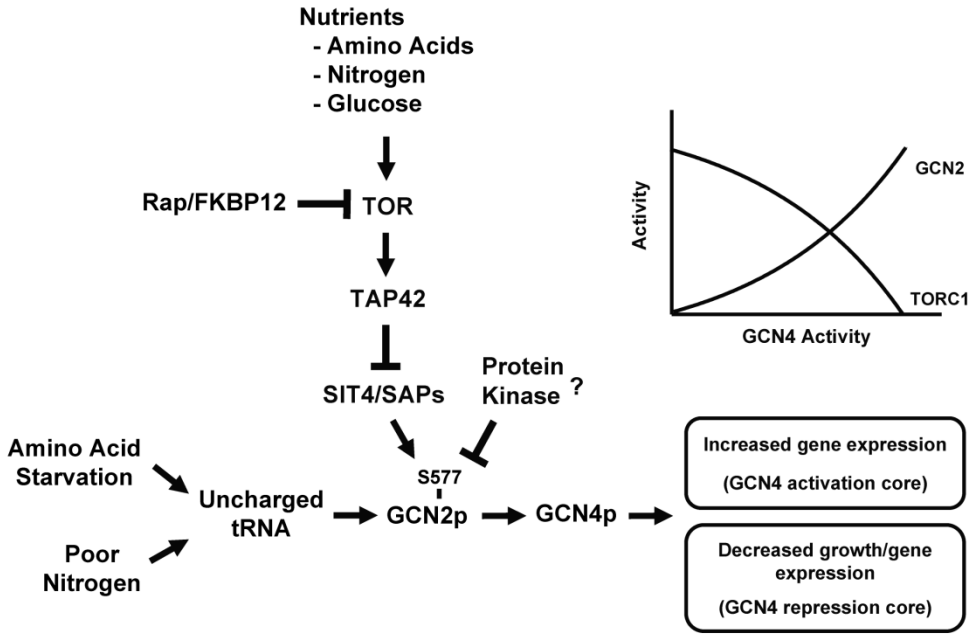


Figure 20. The role of the general amino acid control pathway in TOR-regulated gene expression. Model depicting the role of GAAC in TOR-regulated gene expression including genes in the Gcn4p-dependent activation core and repression core. The *top right panel* illustrates a model wherein enhanced Gcn2p activity contributes to elevated Gcn4p transcriptional function. Conversely, TOR signaling serves to repress the GAAC, with elevated Gcn4p function occurring with lowered TORC1 activity.

nitrogen assimilation of aromatic amino acids, by a mechanism involving activation of *ARO9* and *ARO10* transcription (Figs. 13A-C).

A role for Gcn4p in nitrogen catabolism was also suggested by Gonzalez and colleagues, who were the first to show that *GCN4* translation can be induced by rapamycin (16). Gcn4p was suggested to contribute to the transcription of Gln3p-targeted genes, *DAL1* and *DAL5*, which are important for uptake and degradation of the nitrogen source allantoin, and for *UGA3*, a focus of this study (16,184). These findings further support the idea that the transcription of genes involved in nitrogen assimilation can be coordinately regulated by Gcn4p and Gln3p, as illustrated in Fig. 19E.

IV. Future Directions

This work discovers that there are new changes in the yeast transcriptome following amino acid starvation or inactivation of the TOR signaling pathway with rapamycin and these findings raise several interesting questions. First, while we show that Gcn4p functions to induce the transcription of a core set of genes, the GAAC retains the ability to increase gene expression in a stress-specific fashion. How does Gcn4p attain this specificity? As described in this dissertation, one possible mechanism is that Gcn4p functions coordinately with other transcription factors, such as Gln3p to increase transcription of genes involved in the utilization of secondary nitrogen sources such as GABA. It is possible that Gcn4p functions in conjunction with additional transcription regulators to achieve stress-specific induction of genes expression. A second possible mechanism contributing to the specificity of Gcn4p transcriptional activity involves post-translational modifications that may change the expression of Gcn4p-targeted genes.

Gcn4p is known to be phosphorylated on multiple residues and subject to ubiquitin-mediated degradation (73,171,177). Whether or not these Gen4p control processes are regulated in response to stress is not known. Along these lines, we noted a significant increase in Gcn4p transcriptional activity in the absence of Gcn2p protein kinase following growth in the alternative nitrogen source GABA (Fig. 15C). Experiments to address the effects of stress on DNA binding versus the activation function of Gcn4p may shed light on whether stress-specific signaling plays a role in Gcn4p function.

In addition to *UGA3*, several uncharacterized genes were shown to be co-regulated by Gcn4p and Gln3p (Table 5). These include *YKL050C*, *YIL165C*, YHR029C, *YDL032W*, *RTC2*, *YNR068C*, and *YMR323W*. It is tempting to speculate that many of these genes might play a role in nitrogen metabolism. Gene deletion experiments followed by growth tests in the presence of alternative nitrogen sources could be carried out to address this possibility.

In addition to its role in the activation of transcription, Gcn4p contributes to the repression of genes involved in protein synthesis such as ribosomal protein genes, as well as genes involved in protein folding, targeting, and sorting, and processing. We have noted that several of these genes containing recognizable GCRE motifs in their promoter regions. Whether or not direct DNA binding by Gcn4p in the context of these promoters contributes to gene repression is not known, and it would be of interest to address the underlying mechanisms by which the GAAC can directly or indirectly repress transcription.

V. Summary

The experiments described in this dissertation attempt to address three central questions underlying the integration of the GAAC and TOR signaling pathways. First, is the transcriptome directed by Gcn4p in response to amino acid starvation similar to that in response to inactivation of the TOR signaling pathway by rapamycin treatment, or do different stress conditions allow for significant variations in GCN4p-directed transcription? While we found a large number of genes induced by either 3-AT or rapamycin treatment, a core set of 57 genes was shown to be activated by Gcn4p by either stress condition. The GAC included genes involved in amino acid biosynthesis, nitrogen utilization, signaling, and gene expression. These results indicate that the GAAC has a focal set of genes that are central for its biological function in response to different stresses, but Gcn4p also retains versatility to direct stress-specific gene expression as illustrated by the large collection of genes induced specifically by 3-AT or rapamycin treatment (Fig. 10A).

The second question addressed in this dissertation concerns the nature of the regulatory linkages between the GAAC and TOR pathways. The GAAC and TOR pathways can intersect at multiple points to regulate the transcriptome. In response to rapamycin treatment, we find that Gcn4p is a major transcriptional effector of the TOR pathway, with the number of genes requiring Gcn4p being similar to that dependent on the canonical Gln3p transcription factor (Table 4). Like the Gln3p-directed transcriptome, Gcn4p targets many genes that are central for nitrogen utilization. Rapamycin inhibition of TORC1 is suggested to reduce inhibitory phosphorylation of Gcn2p by activating type 2A-related phosphatases, such as Sit4p, and by inhibiting an

unknown protein kinase (14). Our study suggests that the addition of secondary nitrogen sources, such as GABA, to the medium releases TORC1 inhibition of Gcn2p and the GAAC. Growth in GABA medium also increased uncharged tRNA thus TORC1 signaling and binding of uncharged tRNA to Gcn2p appear to act in concert to phosphorylate eIF2α (Fig. 18). A second point of intersection occurs downstream in the TOR and GAAC pathways. The transcriptional activators Gcn4p and Gln3p can coordinately increase the transcription of target genes, such as *UGA3*, by binding at different UAS elements within their respective promoters (Fig. 19E).

The third question addressed in this work is whether Gcn2p can regulate the transcriptome independent of Gcn4p? With few exceptions, the genes requiring Gcn2p for induction in response to either rapamycin or 3-AT were also dependent on Gcn4p. These results support the model in which the primary role of Gcn2p in yeast is to enhance Gcn4p expression in response to stress. Conversely, almost a third of the genes targeted by Gcn4p displayed significantly reduced requirements for Gcn2p, suggesting that basal levels of Gcn4p retain important biological functions (Fig. 10A, Table 4, and supplemental Table S1 (153)). This idea is consistent with the findings that the *gcn4*Δ cells have a growth defect in the absence of supplemented amino acids, while loss of *GCN2* in an otherwise prototrophic strain does not have a phenotype in SD medium devoid of amino acids. Like Gcn4p, the basal activity of Gln3p is also suggested to have activating functions in the transcriptome.

REFERENCES

1. Wek, R. C. (1994) *Trends in Biochemical Sciences* **19**, 491-496
2. Hinnebusch, A. G. (ed) (2000) *Translational Control of Gene Expression*, Cold Spring Harbor Press, Cold Spring Harbor, NY
3. Kapp, L. D., and Lorsch, J. R. (2004) *Annu Rev Biochem* **73**, 657-704
4. Maag, D., Fekete, C. A., Gryczynski, Z., and Lorsch, J. R. (2005) *Mol Cell* **17**, 265-275
5. Hinnebusch, A. G. (2000) Mechanism and regulation of initiator methionyl-tRNA binding to ribosomes. in *Translational control of gene expression* (Sonenberg, N., Hershey, J. W. B., and Mathews, M. eds.), Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York. pp 185-244
6. Chen, J. J. (2007) *Blood* **109**, 2693-2699
7. Chen, J. J., Crosby, J. S., and London, I. M. (1994) *Biochimie* **76**, 761-769
8. Ron, D., and Walter, P. (2007) *Nat Rev Mol Cell Biol* **8**, 519-529
9. Schroder, M., and Kaufman, R. J. (2005) *Annu Rev Biochem* **74**, 739-789
10. Wek, R. C., Jiang, H. Y., and Anthony, T. G. (2006) *Biochem Soc Trans* **34**, 7-11
11. Garcia, M. A., Gil, J., Ventoso, I., Guerra, S., Domingo, E., Rivas, C., and Esteban, M. (2006) *Microbiol Mol Biol Rev* **70**, 1032-1060
12. Sadler, A. J., and Williams, B. R. (2007) *Curr Top Microbiol Immunol* **316**, 253-292
13. Wek, R. C., Staschke, K. A., and Narasimhan, J. (2004) Regulation of the yeast general amino acid control pathway in response to nutrient stress. in *Nutrient-induced responses in eukaryotic cells* (Winderickx J and Taylor, P. M. ed.), Springer-Verlag, Berlin Heidelberg. pp 171-199
14. Cherkasova, V. A., and Hinnebusch, A. G. (2003) *Genes Dev* **17**, 859-872
15. Narasimhan, J., Staschke, K. A., and Wek, R. C. (2004) *J Biol Chem* **279**, 22820-22832
16. Valenzuela, L., Aranda, C., and Gonzalez, A. (2001) *Journal of Bacteriology* **183**, 2331-2334
17. Hinnebusch, A. G. (1997) *Journal of Biological Chemistry* **272**, 21661-21664
18. Hinnebusch, A. G. (2005) *Annu Rev Microbiol* **59**, 407-450
19. Wek, R. C., Staschke, K. A., and Narasimhan, J. (2004) *Topics in current genetics: Nutrient-induced responses in eukaryotic cells*, Springer Berlin / Heidelberg
20. Hinnebusch, A. G., and Natarajan, K. (2002) *Eukaryot Cell* **1**, 22-32
21. Wek, S. A., Zhu, S., and Wek, R. C. (1995) *Mol Cell Biol* **15**, 4497-4506
22. Zaborske, J. M., Narasimhan, J., Jiang, L., Wek, S. A., Dittmar, K. A., Freimoser, F., Pan, T., and Wek, R. C. (2009) *J Biol Chem* **284**, 25254-25267
23. Dever, T. E., Feng, L., Wek, R. C., Cigan, A. M., Donahue, T. F., and Hinnebusch, A. G. (1992) *Cell* **68**, 585-596
24. Wek, R. C., Jackson, B. M., and Hinnebusch, A. G. (1989) *Proc Natl Acad Sci U.S.A.* **86**, 4579-4583
25. Zhu, S., Sobolev, A. Y., and Wek, R. C. (1996) *Journal of Biological Chemistry* **271**, 24989-24994

26. Dong, J., Qiu, H., Garcia-Barrio, M., Anderson, J., and Hinnebusch, A. G. (2000) *Mol Cell* **6**, 269-279
27. Qiu, H., Dong, J., Francklyn, C. S., and Hinnebusch, A. G. (2001) *EMBO J* **20**, 1425-1438
28. Qiu, H., Garcia-Barrio, M. T., and Hinnebusch, A. G. (1998) *Mol Cell Biol* **18**, 2697-2711
29. Qiu, H., Hu, C., Dong, J., and Hinnebusch, A. G. (2002) *Genes Dev* **16**, 1271-1280
30. Garriz, A., Qiu, H., Dey, M., Seo, E. J., Dever, T. E., and Hinnebusch, A. G. (2009) *Mol Cell Biol* **29**, 1592-1607
31. Romano, P. R., Garcia-Barrio, M. T., Zhang, X., Wang, Q., Taylor, D. R., Zhang, F., Herring, C., Mathews, M. B., Qin, J., and Hinnebusch, A. G. (1998) *Molecular and Cellular Biology* **18**, 2282-2297
32. Wek, R. C., Cannon, J. F., Dever, T. E., and Hinnebusch, A. G. (1992) *Molecular and Cellular Biology* **12**, 5700-5710
33. Connor, J. H., Weiser, D. C., Li, S., Hallenbeck, J. M., and Shenolikar, S. (2001) *Molecular and Cellular Biology* **21**, 6841-6850
34. Novoa, I., Zeng., H., Harding, H. P., and Ron, D. (2001) *Journal of Cellular Biology* **153**, 1011-1022
35. Novoa, I., Zhang, Y., Zeng, H., Jungreis, R., Harding, H. P., and Ron, D. (2003) *EMBO Journal* **22**, 1180-1187
36. Engelberg, D., Klein, C., Marinetto, H., Struhl, K., and Karin, M. (1994) *Cell* **77**, 381-390
37. Vazquez de Aldana, C. R., Wek, R. C., Segundo, P. S., Truesdell, A. G., and Hinnebusch, A. G. (1994) *Molecular and Cellular Biology* **14**, 7920-7932
38. Qiu, H., Hu, J., Anderson, J., Bjork, G. R., Sarkar, S., Hopper, A. K., and Hinnebusch, A. G. (2000) *Mol Cell Biol* **20**, 2505-2516
39. Ramirez, M., Wek, R. C., and Hinnebusch, A. G. (1991) *Mol Cell Biol* **11**, 3027-3036
40. Zhu, S., and Wek, R. C. (1998) *Journal of Biological Chemistry* **273**, 1808-1814
41. Garcia-Barrio, M., Dong, J., Ufano, S., and Hinnebusch, A. G. (2000) *EMBO J* **19**, 1887-1899
42. Kubota, H., Ota, K., Sakaki, Y., and Ito, T. (2001) *J Biol Chem* **276**, 17591-17596
43. Kubota, H., Sakaki, Y., and Ito, T. (2000) *Journal of Biological Chemistry* **275**, 20243-20246
44. Marton, M. J., Vazquez de Aldana, C. R., Qui, H., Chakraburtty, K., and Hinnebusch, A. G. (1997) *Molecular and Cellular Biology* **17**, 4474-4489
45. Yang, R., Wek, S. A., and Wek, R. C. (2000) *Mol Cell Biol* **20**, 2706-2717
46. Vattem, K., Staschke, K. A., and Wek, R. C. (2001) *European Journal of Biochemistry* **268**, 3674-3684
47. Sattlegger, E., and Hinnebusch, A. G. (2000) *EMBO Journal* **19**, 6622-6633
48. Murchie, M. J., and Leader, D. P. (1978) *Biochemica et Biophysica Acta* **520**, 233-236
49. Marton, M. J., Crouch, D., and Hinnebusch, A. G. (1993) *Molecular and Cellular Biology* **13**, 3541-3556

50. Vazques de Aldana, C. R., Marton, M. J., and Hinnebusch, A. G. (1995) *EMBO Journal* **14**, 3184-3199
51. Pereira, C. M., Sattlegger, E., Jiang, H. Y., Longo, B. M., Jaqueta, C. B., Hinnebusch, A. G., Wek, R. C., Mello, L. E., and Castilho, B. A. (2005) *Journal of Biological Chemistry* **280**, 28316-28323
52. Sattlegger, E., Swanson, M. J., Ashcraft, E. A., Jennings, J. L., Fekete, R. A., Link, A. J., and Hinnebusch, A. G. (2004) *J Biol Chem* **279**, 29952-29962
53. Rowlands, A. G., Panniers, R., and Henshaw, E. C. (1988) *Journal of Biological Chemistry* **263**, 5526-5533
54. Dever, T. E., Yang, W., Astrom, S., Bystrom, A. S., and Hinnebusch, A. G. (1995) *Mol Cell Biol* **15**, 6351-6361
55. Pravitt, G. D., Ramiah, K. V. A., Kimball, S. R., and Hinnebusch, A. G. (1998) *Genes and Development* **12**, 514-526
56. Krishnamoorthy, T., Pavitt, G. D., Zhang, F., Dever, T. E., and Hinnebusch, A. G. (2001) *Molecular and Cellular Biology* **21**, 5018-5030
57. Gaba, A., Wang, Z., Krishnamoorthy, T., Hinnebusch, A. G., and Sachs, M. S. (2001) *EMBO Journal* **20**, 6453-6463
58. Grant, C. M., and Hinnebusch, A. G. (1994) *Molecular and Cellular Biology* **14**, 606-618
59. Ashe, M. P., De Long, S. K., and Sachs, A. B. (2000) *Molecular Biology of the Cell* **11**, 833-848
60. Wek, R. C., Ramirez, M., Jackson, B. M., and Hinnebusch, A. G. (1990) *Molecular and Cellular Biology* **10**, 2820-2831
61. Ramirez, M., Wek, R. C., Vazquez de Aldana, C. R., Jackson, B. M., Freeman, B., and Hinnebusch, A. G. (1992) *Molecular and Cellular Biology* **12**, 5801-5815
62. Dever, T. E., Chen, J. J., Barber, G. N., Cigan, A. M., Feng, L., Donahue, T. F., London, I. M., Katze, M. G., and Hinnebusch, A. G. (1993) *Proc Natl Acad Sci U.S.A.* **90**, 4616-4620
63. Sood, R., Porter, A. C., Ma, K., Quilliam, L. A., and Wek, R. C. (2000) *Biochem J* **346**, 281-293
64. Tripahti, G., Wiltshire, C., Macaskill, S., Tournu, H., Budge, S., and Brown, A. J. P. (2002) *EMBO Journal* **21**, 5448-5456
65. Paluh, J. L., Orback, M. J., Legerton, T. L., and Yanofsky, C. (1988) *Proceedings of the National Academy of Sciences, U.S.A.* **85**, 3728-2732
66. Luo, Z., Freitag, M., and Sachs, M. S. (1995) *Molecular and Cellular Biology* **15**, 5235-5245
67. Harding, H. P., Novoa, I., Zhang, Y., Zeng, H., Wek, R., Schapira, M., and Ron, D. (2000) *Mol Cell* **6**, 1099-1108
68. Vattem, K. M., and Wek, R. C. (2004) *Proc Natl Acad Sci U S A* **101**, 11269-11274
69. Hoffmann, B., Valerius, O., Andermann, M., and Braus, G. H. (2001) *Molecular Biology of the Cell* **12**, 2846-2857
70. Wilusz, C. J., Wang, W., and Peltz, S. W. (2001) *Genes and Development* **15**, 2781-2785
71. Ruiz-Echevarria, M. J., and Peltz, S. W. (2000) *Cell* **101**, 741-751

72. Pries, R., Bomeke, K., Irniger, S., Grundmann, O., and Braus, G. H. (2002) *Eukaryotic Cell* **1**, 663-672
73. Meimoun, A., Holtzman, T., Weissman, Z., McBride, H. J., Stillman, D. J., Fink, G. R., and Kornitzer, D. (2000) *Molecular Biology of the Cell* **11**, 915-927
74. Shemer, R., Meimoun, A., Holtzman, T., and Kornitzer, D. (2002) *Molecular and Cellular Biology* **22**, 5395-5404
75. Chi, Y., Huddlerston, M. J., Zhang, X., Young, R. A., Annan, R. S., Carr, S. A., and Deshaies, R. J. (2001) *Genes and Development* **15**, 1078-1092
76. Hinnebusch, A. G. (1992) General and pathway-specific regulatory mechanisms controlling the synthesis of amino acid biosynthetic enzymes in Saccharomyces cerevisiae. in *The molecular and cellular biology of the yeast Saccharomyces* (Jone, E. W., Pringle, J. R., and Broach, J. R. eds.), Cold Spring Harbor Laboratory Press, Plainview, N.Y. pp 319-414
77. Hai, T., and Hartman, M. G. (2001) *Gene* **273**, 1-11
78. Jia, M. H., Larossa, R. A., Lee, J. M., Rafalski, A., Derose, E., Gonye, G., and Xue, Z. (2000) *Physiol Genomics* **3**, 83-92
79. Natarajan, K., Meyer, M. R., Jackson, B. M., Slade, D., Roberts, C., Hinnebusch, A. G., and Marton, M. J. (2001) *Mol Cell Biol* **21**, 4347-4368
80. Jackson, B. M., Drysdale, C. M., Natarajan, K., and Hinnebusch, A. G. (1996) *Molecular and Cellular Biology* **16**, 5557-5571
81. Drydale, C. M., Jackson, B. M., McVeigh, R., Klebanow, E. R., Bai, Y., Kokubo, T., Swanson, M., Nakatani, Y., Weil, P. A., and Hinnebusch, A. G. (1998) *Molecular and Cellular Biology* **18**, 1711-1724
82. Swanson, M. J., Qiu, H., Sumibcay, L., Krueger, A., Kim, S. J., Natarajan, K., Yoon, S., and Hinnebusch, A. G. (2003) *Mol Cell Biol* **23**, 2800-2820
83. Berger, S. L., and Sterner, D. E. (2000) *Microbiology and Molecular Biology Review* **64**, 435-459
84. Natarajan, K., Jackson, B. M., Zhou, H., Winston, F., and Hinnebusch, A. G. (1999) *Molecular Cell* **4**, 657-664
85. Chua, G., Morris, Q. D., Sopko, R., Robinson, M. D., Ryan, O., Chan, E. T., Frey, B. J., Andrews, B. J., Boone, C., and Hughes, T. R. (2006) *Proc Natl Acad Sci U S A* **103**, 12045-12050
86. Moehle, C. M., and Hinnebusch, A. G. (1991) *Molecular and Cellular Biology* **11**, 2723-2735
87. Neuman-Silberberg, F. S., Bhattacharya, S., and Broach, J. R. (1995) *Molecular and Cellular Biology* **15**, 3187-3196
88. Li, B., Nierra, C. R., and Warner, J. R. (1999) *Molecular and Cellular Biology* **19**, 5393-5404
89. Schmelzle, T., and Hall, M. N. (2000) *Cell* **103**, 253-262
90. Crespo, J. L., Powers, T., Fowler, B., and Hall, M. N. (2002) *Proc Natl Acad Sci U S A* **99**, 6784-6789
91. Geraght, M. T., Bassett, D., Morrell, J. C., Gatto, G. J., Bai, J., Geisbrecht, B. V., Hieter, P., and Gould, S. J. (1999) *Proceedings of the National Academy of Sciences, USA* **96**, 2937-2942
92. Rolfes, R. J., and Hinnebusch, A. G. (1993) *Molecular and Cellular Biology* **13**, 5099-5111

93. Springer, C., Kunzler, M., Balmelli, T., and Braus, G. H. (1996) *Journal of Biological Chemistry* **271**, 29637-29643
94. Cashel, M., and Rudd, K. E. (1987) *Escherichia coli and Salmonella typhimurium: cellular and molecular biology*, 1410-1438
95. Miyamoto, Y., Machida, K., Mizunuma, M., Emoto, Y., Sato, N., Miyahara, K., Hirata, D., Usui, T., Takahashi, H., Osada, H., and Miyakawa, T. (2002) *J Biol Chem* **277**, 28810-28814
96. Reggiori, F., and Klionsky, D. J. (2002) *Eukaryotic Cell* **1**, 11-21
97. Talloczy, Z., Jiang, W., Virgin, H. W., Leib, D. A., Scheuner, D., Kaufman, R. J., Eskelinen, E.-L., and Levine, B. (2002) *Proceedings of the National Academy of Sciences U.S.A.* **99**, 190-195
98. Takeshig, K., Baba, M., Tsuboi, S., Noda, T., and Ohsumi, Y. (1992) *Journal of Cell Biology* **119**, 301-311
99. Grundmann, O., Mosch, H. U., and Braus, G. H. (2001) *J Biol Chem* **276**, 25661-25671
100. Francois, J., and Parrou, J. L. (2001) *FEMS Microbiology Reviews* **25**, 125-145
101. Kohlhaw, G. B. (2002) *Microbiology and Molecular Biology Reviews* **67**, 1-15
102. Messenguy, F., Colin, D., and ten Have, J. P. (1980) *Eur J Biochem* **108**, 439-447
103. Hoffmann, B., Wanke, C., LaPagia, S. K., and Braus, G. H. (2000) *Molecular Microbiology* **37**, 28-41
104. Goosens, A., Dever, T. E., Pascual-Ahuir, A., and Serrano, R. (2001) *J Biol Chem* **276**, 30753-30760
105. Palmer, L. K., Wolfe, D., Keeley, J. L., and Keil, R. L. (2002) *Genetics* **161**, 563-574
106. Rodriguez-Hernandez, C. J., Sanchez-Perez, I., Gil-Mascarell, R., Rodriguez-Afonso, A., Torres, A., Perona, R., and Murguia, J. R. (2003) *J Biol Chem* **278**, 33887-33895
107. Crespo, J. L., and Hall, M. N. (2002) *Microbiology and Molecular Biology Reveiws* **66**, 579-591
108. Jelinksy, S. A., and Samson, L. D. (1999) *Proceedings of the National Academy of Science, U.S.A.* **96**, 1486-1491
109. Nishitoh, H., Matsuzawa, A., Tobiume, K., Saegusa, K., Takeda, K., Inoue, K., Hori, S., Kakizuka, A., and Ichijo, H. (2002) *Genes and Development* **16**, 1345-1355
110. Bertolotti, A., Zhang, Y., Hendershot, L. M., Harding, H. P., and Ron, D. (2000) *Nature Cell Biology* **2**, 326-332
111. Ma, K., Vattem, K. M., and Wek, R. C. (2002) *Journal of Biological Chemistry* **277**, 18728-18735
112. Donze, O., and Picard, D. (1999) *Mol Cell Biol* **19**, 8422-8432
113. Stitzel, M. L., Durso, R., and Reese, J. C. (2001) *Genes and Development* **15**, 128-133
114. Harding, H. P., Calfon, M., Urano, F., Novoa, I., and Ron, D. (2002) *Ann Rev Cell Dev Biol* **18**, 575-599
115. Kaufman, R. J., Scheuner, D., Schroder, M., Shen, X., Lee, K., Lin, C. Y., and Arnold, S. M. (2002) *Nature Review Molecular Cell Biology* **3**, 411-421

116. Sidrauski, C., Chapman, R., and Walter, P. (1998) *Trends in Cellular Biology* **8**, 245-249
117. Travers, K. J., Patil, C. K., Wodicka, L., Lockhart, D. J., Weissman, J. S., and Walter, P. (2000) *Cell* **101**, 249-258
118. Martin, D. E., and Hall, M. N. (2005) *Curr Opin Cell Biol* **17**, 158-166
119. Wullschleger, S., Loewith, R., and Hall, M. N. (2006) *Cell* **124**, 471-484
120. Keith, C. T., and Schreiber, S. L. (1995) *Science* **270**, 50-51
121. De Virgilio, C., and Loewith, R. (2006) *Int J Biochem Cell Biol* **38**, 1476-1481
122. Jacinto, E., Loewith, R., Schmidt, A., Lin, S., Ruegg, M. A., Hall, A., and Hall, M. N. (2004) *Nat Cell Biol* **6**, 1122-1128
123. Loewith, R., Jacinto, E., Wullschleger, S., Lorberg, A., Crespo, J. L., Bonenfant, D., Oppliger, W., Jenoe, P., and Hall, M. N. (2002) *Mol Cell* **10**, 457-468
124. Sarbassov, D. D., Ali, S. M., Kim, D. H., Guertin, D. A., Latek, R. R., Erdjument-Bromage, H., Tempst, P., and Sabatini, D. M. (2004) *Curr Biol* **14**, 1296-1302
125. Urban, J., Soulard, A., Huber, A., Lippman, S., Mukhopadhyay, D., Deloche, O., Wanke, V., Anrather, D., Ammerer, G., Riezman, H., Broach, J. R., De Virgilio, C., Hall, M. N., and Loewith, R. (2007) *Mol Cell* **26**, 663-674
126. Kamada, Y., Fujioka, Y., Suzuki, N. N., Inagaki, F., Wullschleger, S., Loewith, R., Hall, M. N., and Ohsumi, Y. (2005) *Mol Cell Biol* **25**, 7239-7248
127. Dann, S. G., Selvaraj, A., and Thomas, G. (2007) *Trends Mol Med* **13**, 252-259
128. Beck, T., and Hall, M. N. (1999) *Nature* **402**, 689-692
129. Cardenas, M. E., Cutler, N. S., Lorenz, M. C., Di Como, C. J., and Heitman, J. (1999) *Genes and Development* **13**, 3271-3279
130. Cooper, T. G. (1982) Nitrogen metabolism in Saccharomyces cerevisiae. in *The molecular biology of the yeast Saccharmyces cerevisiae: metabolism and gene expression* (Strathern, J. N., Jones, E. W., Broach, J. R. ed.), Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY. pp 39-99
131. Magasanik, B. (1992) Regulation of Nitrogen Utilization. in *The Molecular and Cellular Biology of the Yeast Saccharomyces* (Jones, E. W., Pringle, J. R., and Broach, J. R. eds.), Cold Spring Harbor Laboratory Press. pp 283-318
132. Wiame, J. M., Grenson, M., and Arst, H. N., Jr. (1985) *Adv Microb Physiol* **26**, 1-88
133. De Virgilio, C., and Loewith, R. (2006) *Oncogene* **25**, 6392-6415
134. Marion, R. M., Regev, A., Segal, E., Barash, Y., Koller, D., Friedman, N., and O'Shea, E. K. (2004) *Proc Natl Acad Sci U S A* **101**, 14315-14322
135. Magasanik, B., and Kaiser, C. A. (2002) *Gene* **290**, 1-18
136. Guertin, D. A., and Sabatini, D. M. (2007) *Cancer Cell* **12**, 9-22
137. Anthony, T. G., McDaniel, B. J., Byerley, R. L., McGrath, B. C., Cavener, D. R., McNurlan, M. A., and Wek, R. C. (2004) *J Biol Chem* **279**, 36553-36561
138. Reiling, J. H., and Sabatini, D. M. (2006) *Oncogene* **25**, 6373-6383
139. Ozcan, U., Ozcan, L., Yilmaz, E., Duvel, K., Sahin, M., Manning, B. D., and Hotamisligil, G. S. (2008) *Mol Cell* **29**, 541-551
140. Cully, M., Genevet, A., Warne, P., Treins, C., Liu, T., Bastien, J., Baum, B., Tapon, N., Leevers, S. J., and Downward, J. (2009) *Mol Cell Biol*
141. Hardy, T. A., and Roach, P. J. (1993) *Journal of Biological Chemistry* **268**, 23799-23805

142. Wach, A. (1996) *Yeast* **12**, 259-265
143. Brachmann, C. B., Davies, A., Cost, G. J., Caputo, E., Li, J., Hieter, P., and Boeke, J. D. (1998) *Yeast* **14**, 115-132
144. Burke, D., Dawson, D., and Stearns, T. (2000) *Methods in Yeast Genetics*, Cold Spring Harbor Laboratory Press
145. Mueller, P. P., and Hinnebusch, A. G. (1986) *Cell* **45**, 201-207
146. Sikorski, R. S., and Hieter, P. (1989) *Genetics* **122**, 19-27
147. Albrecht, G., Mosch, H. U., Hoffmann, B., Reusser, U., and Braus, G. H. (1998) *J Biol Chem* **273**, 12696-12702
148. Stanbrough, M., Rowen, D. W., and Magasanik, B. (1995) *Proc Natl Acad Sci U S A* **92**, 9450-9454
149. Mumberg, D., Muller, R., and Funk, M. (1995) *Gene* **156**, 199-122
150. Schmitt, M. E., Brown, T. A., and Trumpower, B. L. (1990) *Nucleic Acids Res* **18**, 3091-3092
151. Zar, J. H. (1999) *Biostatistical Analysis*, Prentice Hall, Upper Saddle River, NJ
152. Benjamini, Y., and Hochberg, Y. (1995) *J R Stat Soc B* **57**, 289-300
153. Staschke, K. A., Dey, S., Zaborske, J. M., Palam, L. R., McClintick, J. N., Pan, T., Edenberg, H. J., and Wek, R. C. (2010) *J Biol Chem*
154. Chen, H., and Fink, G. R. (2006) *Genes Dev* **20**, 1150-1161
155. van Helden, J. (2003) *Nucleic Acids Res* **31**, 3593-3596
156. Harbison, C. T., Gordon, D. B., Lee, T. I., Rinaldi, N. J., Macisaac, K. D., Danford, T. W., Hannett, N. M., Tagne, J. B., Reynolds, D. B., Yoo, J., Jennings, E. G., Zeitlinger, J., Pokholok, D. K., Kellis, M., Rolfe, P. A., Takusagawa, K. T., Lander, E. S., Gifford, D. K., Fraenkel, E., and Young, R. A. (2004) *Nature* **431**, 99-104
157. Ruepp, A., Zollner, A., Maier, D., Albermann, K., Hani, J., Mokrejs, M., Tetko, I., Guldener, U., Mannhaupt, G., Munsterkotter, M., and Mewes, H. W. (2004) *Nucleic Acids Res* **32**, 5539-5545
158. Ashburner, M., Ball, C. A., Blake, J. A., Botstein, D., Butler, H., Cherry, J. M., Davis, A. P., Dolinski, K., Dwight, S. S., Eppig, J. T., Harris, M. A., Hill, D. P., Issel-Tarver, L., Kasarskis, A., Lewis, S., Matese, J. C., Richardson, J. E., Ringwald, M., Rubin, G. M., and Sherlock, G. (2000) *Nat Genet* **25**, 25-29
159. Godard, P., Urrestarazu, A., Vissers, S., Kontos, K., Bontempi, G., van Helden, J., and Andre, B. (2007) *Mol Cell Biol* **27**, 3065-3086
160. Tavazoie, S., Hughes, J. D., Campbell, M. J., Cho, R. J., and Church, G. M. (1999) *Nat Genet* **22**, 281-285
161. Kim, S. K., Lund, J., Kiraly, M., Duke, K., Jiang, M., Stuart, J. M., Eizinger, A., Wylie, B. N., and Davidson, G. S. (2001) *Science* **293**, 2087-2092
162. van Helden, J., Andre, B., and Collado-Vides, J. (1998) *J Mol Biol* **281**, 827-842
163. Bradford, M. M. (1976) *Anal Biochem* **72**, 248-254
164. Varshney, U., Lee, C. P., and TajBhandary, U. L. (1991) *J Biol Chem* **266**, 24712-24718
165. Chan, T. F., Carvalho, J., Riles, L., and Zheng, X. F. (2000) *Proc Natl Acad Sci U S A* **97**, 13227-13232
166. Heitman, J., Movva, N. R., and Hall, M. N. (1991) *Science* **253**, 905-909

167. Gasch, A. P., Spellman, P. T., Kao, C. M., Carmel-Harel, O., Eisen, M. B., Storz, G., Botstein, D., and Brown, P. O. (2000) *Mol Biol Cell* **11**, 4241-4257
168. Crespo, J. L., Helliwell, S. B., Wiederkehr, C., Demougin, P., Fowler, B., Primig, M., and Hall, M. N. (2004) *J Biol Chem* **279**, 37512-37517
169. Kasten, M. M., and Stillman, D. J. (1997) *Mol Gen Genet* **256**, 376-386
170. Andre, B. (1990) *Mol Gen Genet* **220**, 269-276
171. Bomeke, K., Pries, R., Korte, V., Scholz, E., Herzog, B., Schulze, F., and Braus, G. H. (2006) *Mol Biol Cell* **17**, 2952-2962
172. Gey, U., Czupalla, C., Hoflack, B., Rodel, G., and Krause-Buchholz, U. (2008) *J Biol Chem* **283**, 9759-9767
173. Goossens, A., de La Fuente, N., Forment, J., Serrano, R., and Portillo, F. (2000) *Mol Cell Biol* **20**, 7654-7661
174. Wilson, W. A., Wang, Z., and Roach, P. J. (2002) *Mol Cell Proteomics* **1**, 232-242
175. Sentheshanuganathan, S. (1960) *Biochem J* **74**, 568-576
176. Iraqui, I., Vissers, S., Andre, B., and Urrestarazu, A. (1999) *Mol Cell Biol* **19**, 3360-3371
177. Irniger, S., and Braus, G. H. (2003) *Curr Genet* **44**, 8-18
178. Qiu, H., Hu, C., Yoon, S., Natarajan, K., Swanson, M. J., and Hinnebusch, A. G. (2004) *Mol Cell Biol* **24**, 4104-4117
179. Harding, H. P., Zhang, Y., Zeng, H., Novoa, I., Lu, P. D., Calfon, M., Sadri, N., Yun, C., Popko, B., Paules, R., Stojdl, D. F., Bell, J. C., Hettmann, T., Leiden, J. M., and Ron, D. (2003) *Mol Cell* **11**, 619-633
180. Kilberg, M. S., Pan, Y. X., Chen, H., and Leung-Pineda, V. (2005) *Annual Review of Nutrition* **25**, 59-85
181. Wek, R. C., and Cavener, D. R. (2007) *Antioxid Redox Signal* **9**, 2357-2371
182. Govind, C. K., Yoon, S., Qiu, H., Govind, S., and Hinnebusch, A. G. (2005) *Mol Cell Biol* **25**, 5626-5638
183. Scherens, B., Feller, A., Vierendeels, F., Messenguy, F., and Dubois, E. (2006) *FEMS Yeast Res* **6**, 777-791
184. Ishida, C., Aranda, C., Valenzuela, L., Riego, L., Deluna, A., Recillas-Targa, F., Filetici, P., Lopez-Revilla, R., and Gonzalez, A. (2006) *Mol Microbiol* **59**, 1790-1806

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Publications:

1. Vattem, K. M., **Staschke, K. A.**, Zhu, S., and Wek, R.C. (2001) Inhibitory Sequences in the Amino Terminus of the Double-Stranded RNA-Dependent Protein Kinase, PKR, are Important for Regulating Phosphorylation of Eukaryotic Initiation Factor 2α (eIF2α). *Eur. J. Biochem*. **268**, 1143-1153

2. Vattem, K. M., **Staschke, K. A.**, and Wek, R. C. (2001) Mechanism of Activation of PKR: Role of Dimerization and Cellular Localization in the Stimulation of eIF2α Phosphorylation. *Eur. J. Biochem*. **268**, 3674-3684

3. Wek, R. C., **Staschke, K. A.**, and Narasimhan, J. (2004) Regulation of the yeast general amino acid control pathway in response to nutrient stress. in *Topics in current genetics: Nutrient-induced responses in eukaryotic cells*, P. M. Winderickx and J. Taylor (ed.), vol. 7, p. 171-199, Springer-Verlag, Berlin Heidelberg

4. Narasimhan, J., **Staschke, K. A.**, and Wek, R. C. (2004) Dimerization is required for activation of eIF2 kinase GCN2 in response to diverse environmental stress conditions. *J. Biol. Chem*. **279**, 22820-22832

5. Zhou, D., Palam, L. R., Narasimhan, J., **Staschke, K. A.**, and Wek, R. C. (2008) Phosphorylation of eIF2 directs ATF5 translational control in response to diverse stress conditions. *J. Biol. Chem*. **283**, 7064-7073

6. **Staschke, K. A.**, Dey, S., Zaborske, J. M., Palam, L. R., McClintick, J. M., Pan, T., Edenberg, H. J., and Wek, R. C. (2010) Integration of general amino acid control and TOR regulatory pathways in nitrogen assimilation in yeast. *J. Biol. Chem.* In Press

Abstracts and Presentations:

1. Vattem, K. M., **Staschke, K. A.**, and Wek, R. C. Mechanism of Activation of PKR: Role of Inhibitory Sequences, Dimerization, and Cellular Localization in the Stimulation of eIF2α Phosphorylation. Translational Control. Cold Spring Harbor, NY. Sept. 7-10, 2000

2. **Staschke, K. A.**, Vattem, K. M., and Wek, R. C. Role of N-terminal Inhibitory Sequences, Dimerization, and Cellular Localization in the Activation of PKR. Department of Biochemistry & Molecular Biology Retreat, Indiana University School of Medicine. Sept. 9, 2000. Oral Presentation

3. **Staschke, K. A.** The Role of Oligomerization and Cellular Localization in the Regulation of eIF2 Kinases. Post-doc and Graduate Student Seminar Series, Department of Biochemistry & Molecular Biology, Indiana University School of Medicine. March 1, 2001. Oral Presentation

4. Vattem, K. M., **Staschke, K. A.**, and Wek, R. C. Mechanism of Activation of PKR: Role of Inhibitory Sequences, Dimerization, and Cellular Localization in the Stimulation of eIF2 Phosphorylation. Department of Biochemistry & Molecular Biology Retreat, Indiana University School of Medicine. Oct. 6, 2001

5. Vattem, K. M., **Staschke, K. A.**, and Wek, R. C. Role of Dimerization and Cellular Localization in the Stimulation of PKR Phosphorylation of eIF2. 4th West Coast Meeting on mRNA Stability and Translation. Seattle, WA. Oct. 14 – 16, 2001

6. Staschke, K. A., **Vattem, K. M.**, and Wek, R. C. An eIF2 Kinase-Based Method for Monitoring Protein-Protein Interactions and Protein Targeting in Yeast. Yeast Genetics and Molecular Biology Meeting. Madison, WI. July 30 – Aug. 4, 2002. Poster

7. Narasimhan, J., **Staschke, K. A.**, and Wek, R. C. Activation of GCN2 Requires Dimerization by a Proposed Amphipathic Helix. Department of Biochemistry & Molecular Biology Retreat, Indiana University School of Medicine. Sept. 6, 2002

8. Wek, R. C, Ma, K., Vattem, K. M., Narasimhan, J., **Staschke, K. A.**, and Jiang, H-Y. Regulation of eIF2 Kinases in Response to Endoplasmic Reticulum and Nutritional Stresses. Experimental Biology 2003. Translating the Genome. San Diego, CA. 2003

9. **Staschke, K. A.**, Narasimhan, J., and Wek, R. C. Translational Control by eIF2 Kinase GCN2 is an Important Contributor to Remediation of Rapamycin and Salt-Induced Stresses. Experimental Biology 2003. Department of Biochemistry & Molecular Biology Research Day, Indiana University School of Medicine. Aug. 22, 2003. Poster

10. Narasimhan, J., **Staschke, K. A.**, and Wek, R. C. Dimerization is required for activation of eIF2 kinase GCN2 in response to diverse environmental stress conditions. 36th Central Regional Meeting of the American Chemical Society. June 2 – 5, 2004. Abstract #3411

11. Narasimhan, J., **Staschke, K. A.**, and Wek, R. C. Dimerization is required for activation of eIF2 kinase GCN2 in response to diverse environmental stress conditions. Translational Control. Cold Spring Harbor, NY. Sept. 7-12, 2004. Poster

12. **Staschke, K. A.** Cross-talk between the general control and TOR signaling pathways in yeast. Research Seminar Series, Department of Biochemistry and Molecular Biology, Indiana University School of Medicine, Indianapolis, IN. April 28, 2005. Oral Presentation

13. **Staschke, K. A.**, Narasimhan, J., and Wek, R. C. Nitrogen and amino acid sensing pathways converge to mediate sensitivity to the immunosuppressant rapamycin. Biochemistry Retreat, Department of Biochemistry and Molecular Biology, Indiana University School of Medicine, Sept. 30, 2005. Poster #52

14. **Staschke, K. A.**, Narasimhan, J., and Wek, R. C. Transcriptional profiling reveals interplay between general control and TOR signaling pathways. Translational Control, Cold Spring Harbor, NY. Sept. 6 – 10, 2006, Poster

15. Dey, S., **Staschke, K. A.**, and Wek, R. C. The stress responsive transcription factors, Gcn4 and Gln3, are required independently for TOR-regulated expression of the Uga3 transcription factor in yeast. Biochemistry Retreat, Department of Biochemistry and Molecular Biology, Indiana University School of Medicine, Sept. 28, 2007. Poster

16. **Staschke, K. A.** The stress responsive transcription factors, Gcn4 and Gln3, act in concert to regulate nitrogen utilization in yeast. Student Seminar Series, Department of Biochemistry and Molecular Biology, Indiana University School of Medicine, Indianapolis, IN. Nov 14th, 2007. Oral Presentation

17. Zhou, D., Palam, L. R., **Staschke, K. A.**, and Wek, R. C. Phosphorylation of eIF2 directs ATF5 translational control in response to diverse stress conditions. Molecular Chaperones and Stress Responses. Cold Spring Harbor, NY. April 30 – May 4, 2008. Poster

18. **Staschke**, K. A., Dey, S., and Wek, R. C. Integration of general control and nitrogen regulatory pathways during nutrient stress in yeast. Molecular Chaperones and Stress Responses. Cold Spring Harbor, NY. April 30 – May 4, 2008. Poster

19. **Staschke**, K. A., Dey, S., Jiang, L., and Wek, R. C. Activation of GCN2 eIF2 kinase in yeast by alternative nitrogen sources. Translational Control, Cold Spring Harbor, NY. Sept 3 – 7, 2008. Poster #301

20. Zhou, D., Palam, R. L., **Staschke, K. A.**, and Wek, R. C. Phosphorylation of eIF2 directs ATF5 translational control in response to environmental stress. Translational Control, Cold Spring Harbor, NY. Sept 3 – 7, 2008. Poster #340

21. Dey, S., **Staschke, K. A.**, and Wek, R. C. Differential regulation of integrated stress pathway in response to DNA damage. 2008 Biochemistry and Molecular Biology Research Day, Indiana University School of Medicine, Oct. 2, 2008. Poster #35

22. Zhou, D., Palam, L. R., **Staschke**, K. A., and Wek, R. C. Phosphorylation of eIF2 directs ATF5 translational control in response to different stresses. 2008 Biochemistry and Molecular Biology Research Day, Indiana University School of Medicine, Oct. 2, 2008. Poster #11

23. **Staschke**, K. A., Dey, S., Jiang, L., and Wek, R. C. Activation of GCN2 eIF2 kinase in yeast by alternative nitrogen sources. 2008 Biochemistry and Molecular Biology Research Day, Indiana University School of Medicine, Oct. 2, 2008. Poster #49