Global phosphoproteomics pinpoints uncharted Gcn2-mediated mechanisms of translational control

Graphical abstract

Highlights

- Global phosphoproteomics identifies hitherto elusive physiological Gcn2 targets
- Gcn2 phosphorylates eIF2β to promote the GDI function of eIF5 toward eIF2-GDP
- This restricts eIF2 ternary complex recycling in amino-acid-starved cells
- Gcn2 engages in a negative autoregulatory feedback loop by targeting Gcn20

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In brief

The Gcn2 kinase mediates cellular adaptations to amino acid limitation through the translational control of gene expression that is primarily executed by eIF2α phosphorylation. Here, Dokládal et al. show that Gcn2 targets auxiliary, physiologically relevant effectors, including eIF2β and Gcn20, to fine-tune translational control in response to amino acid starvation.
SUMMARY

The conserved Gcn2 protein kinase mediates cellular adaptations to amino acid limitation through translational control of gene expression that is exclusively executed by phosphorylation of the $\alpha$-subunit of the eukaryotic translation initiation factor 2 (eIF2$\alpha$). Using quantitative phosphoproteomics, however, we discovered that Gcn2 targets auxiliary effectors to modulate translation. Accordingly, Gcn2 also phosphorylates the $\beta$-subunit of the trimeric eIF2 G protein complex to promote its association with eIF5, which prevents spontaneous nucleotide exchange on eIF2 and thereby restricts the recycling of the initiator methionyl-tRNA-bound eIF2-GDP ternary complex in amino-acid-starved cells. This mechanism contributes to the inhibition of translation initiation in parallel to the sequestration of the nucleotide exchange factor eIF2B by phosphorylated eIF2$\alpha$. Gcn2 further phosphorylates Gcn20 to antagonize, in an inhibitory feedback loop, the formation of the Gcn2-stimulatory Gcn1-Gcn20 complex. Thus, Gcn2 plays a substantially more intricate role in controlling translation initiation than hitherto appreciated.

INTRODUCTION

Adaptation to changes in extracellular amino acid levels critically define the growth and survival of cells. Eukaryotes use two primordial signaling pathways that allow them to sense and respond to fluctuating levels of amino acids, namely the general control nonderepressible 2 (Gcn2) and the target of rapamycin complex 1 (TORC1) pathways that are activated by the absence and presence, respectively, of amino acids (Albert and Hall, 2015; Hinnebusch, 2005; Wolfson and Sabatini, 2017). Gcn2 senses and is activated by uncharged tRNAs that accumulate when amino acids become limiting (Dong et al., 2000; Zhu et al., 1996). Active Gcn2 phosphorylates the $\alpha$-subunit of the eukaryotic translation initiation factor eIF2 (a G protein complex) to inhibit general translation initiation and activate translational derepression of specific mRNAs (Dever et al., 1992; Hinnebusch, 2005; Wolfson and Sabatini, 2017). Gcn2 senses and is activated by uncharged tRNAs that accumulate when amino acids become limiting (Dong et al., 2000; Zhu et al., 1996). Active Gcn2 phosphorylates the $\alpha$-subunit of the eukaryotic translation initiation factor eIF2 (a G protein complex) to inhibit general translation initiation and activate translational derepression of specific mRNAs (Dever et al., 1992; Hinnebusch, 2005). Mechanistically, guanosine triphosphate (GTP)-bound eIF2 fulfills an essential function for protein synthesis by delivering the initiator methionyl-tRNA (as eIF2-GTP-tRNA$^{Met}$ ternary complex [TC]) to the 40S ribosome, which then scans and selects the translation initiation codon. Gcn2-mediated phosphorylation of eIF2$\alpha$ (eIF2$\alpha$P), however, limits the levels of GTP-bound eIF2 and consequently those of TCs that are available for translation initiation by converting eIF2 from a substrate to an inhibitor of its guanine nucleotide exchange factor (GEF) eIF2B. While this globally dampens translation, it also stimulates the translation of specific mRNAs such as GCN4 in yeast and ATF4 in mammals, which code for transcription factors that induce the expression of a large set of genes involved in the cellular adaptation to amino acid limitation (Hinnebusch, 2005; Kilberg et al., 2009). The respective translational stimulation is based on a particular re-initiation mechanism that allows preinitiation complexes to bypass upstream open reading frames (uORFs) in the 5' untranslated regions (UTRs) of GCN4 and ATF4 mRNAs and reach the main ORF (Mueller and Hinnebusch, 1986; Vattem and Wek, 2004).

The TORC1 kinase is a master regulator of cell growth that couples environmental and nutritional cues to downstream effectors that oppositely control anabolic (e.g., protein synthesis) and catabolic (e.g., macroautophagy) processes (Albert and Hall, 2015; Laplante and Sabatini, 2012). Amino acids signal to TORC1 through various sensory modules and upstream regulators that converge on the conserved heterodimeric Rag guanosine triphosphatases (GTPases) consisting of RagA (or RagB) and RagC (or RagD) in higher eukaryotes and Gtr1 and Gtr2 in yeast (Binda et al., 2009; Kim et al., 2008; Sancak et al., 2008). Amino acid abundance promotes a configuration of the Rag GTPases that activates TORC1 (i.e., RagA/B/Gtr1 GTP and RagC/G/rGtr2 guanosine diphosphate [GDP] loaded), while amino acid starvation converts the Rag GTPases into their opposite loading state that inactivates TORC1 (Binda et al., 2009; Demetriades et al., 2014; Kim et al., 2008; Sancak et al., 2008). Interestingly, the TORC1 and Gcn2 signaling pathways are wired to each other through a feedback regulatory loop in which (1)
TORC1 inhibits Gcn2 (by preventing the dephosphorylation of Gcn2-pSer2377 and thus reducing the affinity of Gcn2 for uncharged tRNAs) and (2) Gcn2 inhibits TORC1 (possibly through phosphorylation of its subunit Kog1) (Cherkasova and Hinnebusch, 2003; Yuan et al., 2017).

Based on global phosphoproteomic studies, we show here that the Gcn2 kinase targets other proteins, in addition to eIF2α, that have hitherto remained elusive. Specifically, Gcn2 phosphorylates the β-subunit of eIF2 (eIF2β or Sui3) to promote its association with the translation initiation factor eIF5 (TIF5). This event is physiologically relevant as Ser to Ala mutation of the Gcn2 target residue in eIF2β compromises its proper association with eIF5 and partially reduces the capacity of cells to inhibit translation initiation when treated with rapamycin or starved for amino acids, without changing their eIF2α-P levels. These data elucidate a missing mechanistic aspect of a model in which amino acid starvation-induced eIF2α-eIF5 complex formation limits the GDP release by and spontaneous nucleotide exchange on eIF2 to restrict TC recycling in parallel to eIF2α-P-mediated sequestration of eIF2B (Jennings and Pavitt, 2010). In addition to eIF2β, Gcn2 also phosphorylates Gcn20. This phosphorylation weakens the Gcn1-Gcn20 complex formation and hence the capacity of this complex to activate Gcn2 by uncharged tRNAs in vivo (Vazquez de Aldana et al., 1995). Thus, our data also highlight the existence of a feedback inhibition loop that modulates Gcn2 activity itself.

RESULTS AND DISCUSSION

Global phosphoproteomics identifies hitherto elusive Gcn2 targets

The only known bona fide target of the protein kinase Gcn2 in yeast is Ser52 of the eukaryotic translation initiation factor eIF2α (Sui2) (Dever et al., 1992). To identify additional Gcn2 target proteins, we performed a set of stable isotope labeling by amino acids in cell culture (SILAC)-based quantitative phosphoproteomic experiments (Figure 1A). Briefly, we compared wild-type (WT) cells to gcn2Δ and sui2552A cells (n = 3), all treated or not treated with rapamycin, which has previously been shown to stimulate eIF2α phosphorylation by Gcn2 (Cherkasova and Hinnebusch, 2003). These experiments led to the identification of 35,401 phosphorys, 32,110 of which could be quantified (Figure 1B). A total of 27,596 sites could be localized to specific amino acid residues (localization probability ≥ 0.75; Olsen et al., 2006) and were normalized to changes in protein abundance. These sites were used for further analyses (Table S1). The rationale for choosing these strains and experimental conditions relied on both positive and negative selection criteria. By comparing WT to gcn2Δ cells, both treated with rapamycin, Gcn2 target sites should be upregulated in WT cells (Figure 1C, positive selection I). In addition, sites should respond positively to rapamycin treatment in WT cells compared to non-treated WT cells based on our recently published rapamycin-sensitive phosphoproteome (Hu et al., 2019; positive selection II). Sui2 phosphorylation by Gcn2 leads to the translational upregulation of the transcription factor Gcn4, which regulates the expression of 500–1,000 different target genes (Figure 1C) (Jia et al., 2000; Natarajan et al., 2001). By comparing rapamycin-treated WT and sui2552A cells, which are defective in Gcn4 upregulation, we further aimed at discriminating proximal Gcn2 effectors from distal effectors that act downstream of Gcn4. All of the phosphosites that were identified as upregulated in WT compared to sui2552A cells were therefore removed from our list of potential direct Gcn2 targets (Figures 1C, negative selection I). Finally, we also compared rapamycin-treated gcn2Δ to non-treated gcn2Δ cells and also removed all of the sites that were rapamycin sensitive in this setting (n = 3, negative selection II). These stringent filter criteria led to a shortlist of 18 of 27,596 phosphosites, including the known target site Sui2-Ser52, that were significantly regulated and fulfilled the criteria of being excellent candidates for Gcn2 target sites (Figure 1D; p < 0.05, >2-fold change, ≥4 data points per site). The amino acid residues surrounding these 18 phosphosites were used to identify a potential Gcn2 phosphorylation site motif (Figure 1E). Accordingly, Gcn2 seems to prefer negatively charged amino acids at positions −2 (glutamic acid: E), +1 (E), and +5 (aspartate: D), and positively charged ones at positions +2 (lysine: K) and +4 (K and arginine: R). The 16 target proteins carrying significantly regulated sites were further analyzed for known protein-protein interactions using STRING DB (Szklarczyk et al., 2019), which allowed us to generate a protein interaction network consisting of Gcn2 and 9 target proteins (Figure 1F). Interestingly, 8 of these 9 proteins are implicated in protein synthesis. Also, a Gene Ontology (GO) term analysis of target proteins led to a significant enrichment of terms related to mRNA translation and translation initiation (Figure 1G). Our unbiased approach confirms the general assumption that Gcn2 has a rather limited target profile, which is dedicated mainly to the control of protein synthesis and pinpoints 15 potential Gcn2 kinase substrates that have hitherto remained elusive.

Gcn2 has been suggested to directly phosphorylate the raptor N-terminal conserved (RNC) domain in Kog1 to mediate TORC1 inhibition in leucine-starved cells (Yuan et al., 2017). Interestingly, using less stringent criteria than that above in analyzing our phosphoproteome dataset, we also identified one residue in Kog1 (i.e., Ser386 within the RNC domain) that appeared to be regulated in a Gcn2-dependent manner in rapamycin-treated cells (Table S1). The phosphorylation status of this residue, however, was similarly affected in gcn2Δ and Sui2552A-expressing cells, which indicates that it is likely controlled by distal Gcn2 effectors (at least in rapamycin-treated cells) and explains why it was not retained in our list of potential proximal Gcn2 targets (Figure 1D). To examine whether Gcn2 may control TORC1 via distal effectors in leucine-starved cells, we asked whether loss of Gcn2 and of its downstream effector Gcn4 would cause a similar defect in TORC1 inactivation upon leucine starvation. This was indeed the case (Figure S1). Thus, we deem it likely that Gcn2 inhibits TORC1 in vivo indirectly through the Gcn4-mediated expression of a protein that inhibits TORC1 signaling, perhaps as in mammalian cells, where Gcn2 sustains mTORC1 inhibition via ATF4-mediated expression of Sestrin2, a leucine sensor that inhibits mTORC1 signaling through GATOR1 (Ye et al., 2015).

Sui3-Ser80 and Gcn20-Thr94/Ser96 are physiologically relevant Gcn2 target residues

To select some of the newly identified potential Gcn2 target residues for further analysis, we applied an additional criterion for
filtering our data, namely, that positive hits should also be significantly regulated by Gcn2 in leucine-starved cells. To this end, we acquired an independent phosphoproteome dataset (in duplicate) using leucine-starved WT and gcn2Δ cells (Table S2). The respective results prompted us to further examine the role of Sui3-Ser80 and Gcn20-Ser95 (together with Gcn20-Thr94), as these residues were, like Sui2-Ser52 (eIF2α-Ser52), among the ones that were most robustly phosphorylated in a Gcn2-dependent manner in both rapamycin-treated (Figure 1D; Table S1) and leucine-starved (>2-fold in both leucine starvation experiments; Table S2) cells. To assess whether Gcn2 may directly phosphorylate Sui3 and/or Gcn20, we submitted the respective recombinant proteins to in vitro kinase assays. Gratifyingly, a hyperactive Gcn2F842L variant and Gcn2, but not kinase-dead Gcn2KD (Gcn2K628R), were able to phosphorylate recombinant Sui3 and Gcn20, respectively, in vitro (Figure 2A). Because the Gcn2 variants were virtually unable to phosphorylate recombinant Sui3S80A or Gcn20T94A,S95A (Figure 2A), we inferred that the respective amino acids represent the most critical Gcn2-modified residues. Mass spectrometry (MS) analyses of in vitro Gcn2-phosphorylated Sui3 and Gcn20 confirmed that Sui3-Ser80, Gcn20-Thr94, and Gcn20-Ser95 were specifically phosphorylated by Gcn2 and not by Gcn2KD (Figure 2B). Kinetic in vitro analyses further indicated that yeast Gcn2F842L and/or human Gcn2 phosphorylate Sui3 and Gcn20 with affinities comparable to or even higher than those for Sui2 (Figure S2). Combined with our in vivo analyses, these data establish Sui3 and Gcn20 as direct Gcn2 targets.

Sui3, the β-subunit of eIF2, and Gcn20, which dimerizes with Gcn1 to activate Gcn2, are both implicated in translation initiation (Hinnebusch, 2005). To study the role, if any, of Gcn2-mediated phosphorylation of Sui3 and Gcn20 for translation initiation in vivo, we next measured the impact of Ser/Thr to Ala (A) or phosphomimetic Glu (E) mutations of the relevant Gcn2 target residues on GCN4-LacZ expression, a sensitive reporter for the activity of translation initiation factors (Hinnebusch, 2005).
**A**

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**C**

![Graph showing GCN4-LacZ activity](legend on next page)

**D**

SD + Arg/Trp 3-AT (100 mM)

![Image showing yeast colonies](legend on next page)

**E**

<table>
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**F**

- **WT**
- **gcn2Δ**
- **sui256SA**
- **sui356MA**
- **gcn20Δ**
- **sui256SA,s56MA**
- **sui356MA,s56MA**
- **gcn20Δ,T94A,S95A**
- **gcn20Δ,T94E,S95E**

![Polysome profiles](legend on next page)

*P/M EXP: 4.93 ± 0.44, P/M RAP: 1.34 ± 0.05
*P/M EXP: 4.61 ± 0.50, P/M RAP: 3.27 ± 0.33
*P/M EXP: 4.73 ± 0.16, P/M RAP: 3.15 ± 0.08
*P/M EXP: 5.07 ± 0.24, P/M RAP: 1.33 ± 0.03
*P/M EXP: 5.06 ± 0.14, P/M RAP: 3.10 ± 0.16
*P/M EXP: 5.00 ± 0.18, P/M RAP: 1.30 ± 0.05
*P/M EXP: 4.59 ± 0.37, P/M RAP: 1.24 ± 0.18
*P/M EXP: 5.01 ± 0.52, P/M RAP: 1.41 ± 0.06

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As previously reported (Cherkasova and Hinnebusch, 2003), GCN4-LacZ expression was very low in exponentially growing WT cells and became strongly derepressed, in a Gcn2- and eIF2αP-dependent manner, following either rapamycin treatment or histidine starvation (imposed by the addition of 3-amino-1,2,4-triazole [3-AT]; Figure 2C). Interestingly, sui3S80A cells were partially but significantly compromised for both rapamycin- and 3-AT-induced derepression of GCN4-LacZ (by >38% and >38%, respectively), which was not the case for rapamycin- and 3-AT-treated sui3S80E cells. Defective derepression of GCN4 translation is associated with 3-AT-sensitive cell growth. Consistent with this, sui3S80A and the other mutants that exhibited a GCN4-LacZ derepression defect (i.e., gcnc24, sui2S52A, and sui2S52A/sui3S80B), but not sui3S80E, were sensitive to 3-AT (Figures 2D and S3). We considered it possible, therefore, that phosphorylation of Ser80 in Sui3 contributes to the global inhibition of translation initiation in rapamycin- or starved cells by promoting eIF2αP levels. However, in contrast to gcnc24 and sui2S52A cells, sui3S80A cells were not impaired in their capacity to phosphorylate eIF2αP or to inhibit general translation initiation (as assayed by determining the polypeptide:monosome ratios [P:M] from polysome profiles) in 3-AT- or rapamycin-treated cells (Figures 2E and 2F). Gcn2-mediated phosphorylation of Sui3 therefore contributes to GCN4 expression through another mechanism (see below).

The combined mutation of Thr54 and Ser95 in Gcn20 to either Ala or Gln yielded the Gcn20T44A,S95A or Gcn20T44E,S95E variants, which significantly enhanced (by 34%) and reduced (by 33%), respectively, the expression of GCN4-LacZ in exponentially growing cells. Similarly, Gcn20T44A,S95A and Gcn20T44E,S95E variants also significantly enhanced (by >14%) and reduced (by >17%), respectively, the expression of GCN4-LacZ in rapamycin- and 3-AT-treated cells (Figure 2C). Neither gcnc24 allele altered the 3-AT sensitivity or the relative eIF2αP levels (Figures 2D and 2E). The latter matched our expectations because the loss of Gcn20 only moderately affected eIF2αP under the same conditions (Figure 2E), although it rendered cells 3-AT sensitive (Figures 2C, 2D, and S3). Notably, in a similar experiment, eIF2αP levels were previously also found to be only mildly repressed in gcn20Δ cells (Vazquez de Aldana et al., 1995). Nevertheless, our polysome profile analyses indicated that Gcn20T44A,S95A-expressing cells exhibited moderately reduced global translation initiation levels in the exponential growth phase and after rapamycin treatment (i.e., 10.6% and 7.6% lower P:M ratios, respectively), while Gcn20T44E,S95E-expressing cells exhibited normal translation initiation levels in the exponential growth phase, but slightly higher global translation initiation levels in rapamycin-treated cells (i.e., a 8.6% higher P:M ratio) (Figure 2F). Thus, although our polysome profile analyses of Gcn20T44A,S95A and Gcn20T44E,S95E-expressing cells are not significantly different from those of WT cells, they are congruent with our GCN4-LacZ expression analyses in that they indicate a moderate, opposite effect of the non-phosphorylatable and phosphomimetic Gcn20 variants on translation initiation. In summary, Sui3-Ser80 and Gcn20-Thr94/Ser95 are bona fide Gcn2 target residues that are relevant for the proper translational control of GCN4 mRNA in vivo, although their contribution to the downregulation of global translation initiation by Gcn2 is subtle.

Gcn2 promotes eIF2α-eIF5 association through phosphorylation of Sui3-Ser80

Interestingly, Ser90 lies between 2 of the 3 lysine-rich boxes in the N terminus of Sui3, which mediate binding to the aromatic and acidic residue-rich bipartite motifs in the C-terminal domain of both elf5 (Tif5) and the catalytic elf2β-subunit Gcd6 (Asano et al., 1999). We therefore speculated that the Gcn2-mediated phosphorylation of this residue may alter the affinity of Sui3 for Tif5 and/or Gcd6. This was the case for the Sui3-Tif5 interaction: a Sui368–89 peptide (including Ser90 and the lysine-rich box 3; Asano et al., 1999), when phosphorylated at Ser90 (pS90-Sui368–89), bound 15.2-fold more tightly to recombinant Tif5 than a non-phosphorylated (Sui368–89) control peptide when assayed in vitro using fluorescence spectroscopy (Figure 3A). Recombinant Gcd6, in contrast, exhibited a lower affinity for both pS90-Sui368–89 and Sui368–89 (Figure 3B). In essence, our in vivo co-immunoprecipitation analyses validated these results.

Accordingly, 3-AT treatment, which stimulates the activity of Gcn2, strongly reinforced (as also reported earlier; Jennings et al., 1995), the Sui3-Tif5 interaction: a Sui368–89 peptide (including Ser90 and the lysine-rich box 3; Asano et al., 1999), when phosphorylated at Ser90 (pS90-Sui368–89), bound 15.2-fold more tightly to recombinant Tif5 than a non-phosphorylated (Sui368–89) control peptide when assayed in vitro using fluorescence spectroscopy (Figure 3A). Recombinant Gcd6, in contrast, exhibited a lower affinity for both pS90-Sui368–89 and Sui368–89 (Figure 3B). In essence, our in vivo co-immunoprecipitation analyses validated these results. Accordingly, 3-AT treatment, which stimulates the activity of Gcn2, strongly reinforced (as also reported earlier; Jennings et al., 1995), the Sui3-Tif5 interaction: a Sui368–89 peptide (including Ser90 and the lysine-rich box 3; Asano et al., 1999), when phosphorylated at Ser90 (pS90-Sui368–89), bound 15.2-fold more tightly to recombinant Tif5 than a non-phosphorylated (Sui368–89) control peptide when assayed in vitro using fluorescence spectroscopy (Figure 3A). Recombinant Gcd6, in contrast, exhibited a lower affinity for both pS90-Sui368–89 and Sui368–89 (Figure 3B). In essence, our in vivo co-immunoprecipitation analyses validated these results.
Figure 3. Gcn2 promotes eIF2-eIF5 association through phosphorylation of eIF2β/Sui3 on Ser80

(A and B) Phosphorylation of Ser80 in Sui368–89 influences its affinity for eIF5/Tif5 and Gcd6 in vitro. The binding affinities of recombinant GST-Tif5 and GST-Gcd6 for the fluorescein-labeled Sui368–89 peptide, phosphorylated (right panels) or not (left panels) on Ser80, were determined by fluorescence quenching. Three independent measurements were performed at room temperature, with standard deviations being <7% (for A) or <15% (for B) for each presented data point. The concentration of Sui368–89 peptide was 500 nM. Dissociation constants (KD; n = 3; ±SD) were calculated using nonlinear asymmetric sigmoidal regressions.

D, relative difference in fluorescence intensity; kCPS, kilo counts per second.

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and Pavitt, 2010) the otherwise weak association between Sui3 and Tif5 observed in exponentially growing cells (Figure 3C). Sui3S80A, in contrast, remained weakly associated with Tif5, even after 3-AT treatment, while the phosphomimetic Sui3S80E variant was able to constitutively and tightly bind Tif5 in exponentially growing cells, notably to the same extent as in 3-AT-treated cells (Figure 3C). In further agreement with our in vitro assays, all 3 Sui3 variants exhibited similar affinities for Gcd6 in exponentially growing and 3-AT-treated cells, with overall slightly reduced associations between Sui3 variants and Gcd6 under the latter condition (Figure 3D). Together with our genetic data, our biochemical data therefore establish a model in which Gcn2 phosphorylates eIF2β/Sui3 at Ser80 to stimulate its association with eIF5/Tif5.

Notably, Tif5 plays a dual role as a GTPase activating protein (GAP) for the eIF2-GTP-tRNAiMet TC during mRNA start site selection and as a GDP dissociation inhibitor (GDI) for eIF2-GDP (Jennings and Pavitt, 2010). Due to the role of Tif5 in start site selection, certain mutant alleles of Tif5, like mutant alleles of its binding partner Sui3, can engage in non-AUG start codon recognition (a phenotype coined suppressor of initiation codon mutation [Sui]). Depending on the type of mutation, these alleles can constitutively derepress (Gcd− phenotype; which is not the case for suid or suid gcn2Δ cells; Figures 2C, 2D, and S3) or prevent the derepression (Gcn− phenotype) of GCN4 mRNA translation (Antony and Alone, 2017; Thakur et al., 2019). To evaluate whether the Sui3S80A/E mutations may affect GCN4 mRNA translation by affecting start codon selectivity, we measured the expression of HIS4-LacZ reporters containing either an AUG or UUG start codon (Donahue and Cigan, 1988). Expression of the Sui3S80A/E alleles, like expression of the Sui2S52A allele alone or combined with Sui3S80A, and like the loss of Gcn2, had little impact on the UUG:AUG ratio in this assay, while the expression of the Tif5G31R allele significantly increased (5-fold) the UUG:AUG ratio, as previously reported (Antony and Alone, 2017; Thakur et al., 2019) (Figure S4). Thus, the mutation of Ser80 in Sui3 does not affect near cognate start codon recognition on the HIS4-lacZ reporter.

The second function of Tif5—its GDI activity—is specifically important in amino acid-starved cells to prevent spontaneous nucleotide exchange on eIF2, and thus to restrict its recycling to TC. This occurs in parallel to the inhibition of the eIF2B-mediated nucleotide exchange through the tethering of eIF2Bxβ to eIF2αP (Krishnamoorthy et al., 2001; Mohammad-Qureshi et al., 2007). The enhanced GDI function of Tif5 toward eIF2-GDP in amino acid-starved cells is eIF2αP independent, requires guiding by a C-terminal domain in eIF2β/Sui3, and relies on the stimulation of eIF2-Tif5 complex formation by a yet- unidentified mechanism (Jennings et al., 2016; Jennings and Pavitt, 2010). In this context, our data provide a mechanistic explanation for these earlier observations; the eIF2-Tif5 association is triggered by Gcn2-dependent phosphorylation of Sui3-Ser80, which enables it to more efficiently recruit Tif5. Based on this model, we predicted that Sui3S80A-expressing cells would be less susceptible to the Tif5-mediated GDI action and therefore exhibit higher TC levels that translate into lower GCN4-LacZ expression and increased 3-AT sensitivity. In agreement with this model, overproduction of Tif5 (from a high-copy plasmid; TIF5S80A), which we assumed would restore the formation of Sui3G31R-Tif5 complexes by mass action, suppressed the 3-AT sensitivity of suidS80Δ cells but not the sensitivity of eIF2αP-deficient cells (i.e., gcn2Δ, suidS80A, and suidS80A suidS80A; Figures 2D, 3E, and S3). To evaluate the importance of Sui3-Ser80 phosphorylation for the inhibition of translation in general, we also used a hyperactive Gcn2F842L allele that severely reduces growth rates by inhibiting global translation initiation (Qiu et al., 2002). Intriguingly, the mutation of Ser80 to Ala in Sui3, but not Ser80 to Glu, suppressed the growth inhibition imposed by the expression of Gcn2F842L to a similar extent as a mutation of Ser80 to Ala in Sui2 or expression of a Tif5W31F allele with reduced affinity for Sui3 (Jennings and Pavitt, 2010) (Figure 3F). Moreover, this suppressive effect conferred by the Sui3S80A allele, which was not
due to reduced eIF2αP levels (Figure 3G), was fully abrogated by the overproduction of Tif5 (Figure 3F). Finally, the expression of Sui3S80A also significantly suppressed the Gcn2F842L-mediated reduction in global translation initiation (Figure 3H). Our results endorse and extend a model in which Gcn2-stimulated eIF2β/Sui3-eIF5/Tif5 complex formation is part of the cellular response to amino acid starvation that contributes to the proper downregulation of translation initiation and consequently derepression of GCN4 translation (Figure 3I).

**Gcn2 engages in feedback inhibition through Gcn20-Thr94/Ser95 phosphorylation**

Formation of the Gcn1-Gcn20 heterodimer is mediated by the N-terminal 118 amino acids of Gcn20 and the eukaryotic translation elongation factor 3 (eEF3)-like (EF3L) domain in Gcn1 (Vazquez de Aldana et al., 1995). Notably, mutation of the EF3L domain in Gcn1 reduces Gcn1-Gcn20 complex formation and confers a Gcn2-phenotype, which is consistent with the proposed role of the Gcn1-Gcn20 heterodimer in stimulating the activation of Gcn2 by uncharged tRNAs (Hinnebusch, 2005).

Because Thr94 and Ser95 of Gcn20 are part of the interface that mediates Gcn1-binding, our data presented above raised the intriguing possibility that Gcn2 may engage in a feedback regulatory circuit by regulating Gcn1-Gcn20 complex formation through the phosphorylation of Gcn20. Our *in vitro* fluorescence spectroscopy studies with recombinant proteins supported this idea, as the affinity between the EF3L domain of Gcn1 (Gcn1EF3L) and Gcn20 and the one between Gcn1EF3L and Gcn20T94A,S95A was 33- and 18-fold higher, respectively, than the one between Gcn1T94A,S95A and Gcn1EF3L (Figure 4A).

In vivo, both Gcn20 and Gcn20T94A,S95A formed stable heterodimers with Gcn1 in exponentially growing cells as expected (Figure 4B). Treatment with 3-AT curtailed the association between Gcn20 and Gcn1, but not the one between Gcn20T94A,S95A and Gcn1, suggesting that Gcn20-dependent phosphorylation of Thr94/Ser95 in Gcn20 antagonizes Gcn20-Gcn1 complex formation. In support of this notion, Gcn20T94A,S95E was compromised for Gcn1 binding in exponentially growing cells and slightly more so in 3-AT-treated cells, which may be explained by the existence of additional Gcn2 target residues that have escaped detection by MS and that play a minor role in modulating the Gcn20-Gcn1 interaction (Figure 4B). Combined, these data therefore advocate a model in which Gcn2 engages in feedback inhibition through the phosphorylation of Gcn20-Thr94/Ser95, which favors disassembly of the Gcn1-Gcn20 complex.

Our analyses of the distal readouts for Gcn2 activity (i.e., GCN4-LacZ expression and polysome profiles) in gcn20T94A,S95A and gcn20T94A,S95E mutants also support this model (Figures 2C and 2F), even though the proximal Gcn2 readout, namely the eIF2αP levels, appeared unchanged in these mutants (Figure 2E).

We deemed it possible, however, that the sensitivity of our immunoblot analyses and/or the dynamic nature of the proposed regulatory loop may preclude proper resolution of the differences in eIF2αP levels, which we expected to be rather moderate (i.e., within the range of 10%). To enhance the sensitivity of this assay, we therefore used the hyperactive Gcn2F842L allele that boosts eIF2αP in exponentially growing cells (Figure 4C). Similar to the loss of Gcn20, expression of Gcn20T94A,S95E, but not expression of Gcn20T84A,S95A, now clearly reduced the Gcn2F842L-induced eIF2αP levels (Figure 4C), which, as inferred from a control experiment using hemagglutinin (HA)-tagged variants of Gcn20 (Figure 4D), was not caused by Gcn20T84A,S95E instability. In parallel, Gcn20T94A,S95E also partially suppressed the slow-growth phenotype and the inhibition of global translation initiation that is mediated by the expression of the Gcn2F842L allele (Figures 3H, 4E, and 4F). These data also perfectly match with the fact that the gcn20-501 mutation was originally isolated as a suppressor of the slow-growth phenotype conferred by a hyperactive Gcn2 allele (Vazquez de Aldana et al., 1995).

Our study highlights that Gcn2 plays a substantially more intricate role in controlling translation initiation than has been previously appreciated. In this context, our phosphoproteome analyses further indicate that Gcn2 may, in parallel to Sui3 and Gcn20 studied here, also target (1) Yar1, which functions as a dedicated chaperone for the ribosomal protein Rps3 (Koch et al., 2012); (2) Yef3, the translation elongation factor 3 (Qin et al., 1987); (3) Sap185, which, in complex with the type 2A-related serine-threonine phosphatase Sit4, modulates TORC1-regulated, Gcn20-dependent translation (Rohde et al., 2004); and (4) Ssd1, an mRNA-binding protein that spatially and temporally controls the translation of specific mRNAs and/or regulates ribosome biogenesis (Jansen et al., 2009; Kurischko et al., 2011; Li et al., 2009) (Figure 1D). These observations warrant future studies that will address the intriguing possibility that the function of Gcn2 expands beyond its currently known role in translation initiation to a more universal role in shaping various aspects of translation in response to the availability of amino acids.

**Limitations of study**

The chosen bottom-up proteomics strategy ensures a maximum of phosphosite identifications given the technical setup. However, due to the proteolytic digestion of proteins before MS analysis, the crosstalk between different phosphosites cannot be studied and differential regulation of specific isoforms cannot be discriminated. As tandem MS (MS/MS) spectra do not always allow the unambiguous localization of specific phosphosites, which led to their exclusion from further analyses according to our data processing pipeline, some biologically meaningful hits may also have been lost in our MS analyses. Lastly, because our MS data did not cover the full repertoire of peptide variants (i.e. non-phosphorylated and singly or doubly phosphorylated peptide variants), we also could not determine the phosphosite occupancy (stoichiometry of phosphorylation) on Sui2, Sui3, and Gcn20.

A potential limitation with respect to the *in vitro* protein kinase assays that were carried out with the hyperactive Gcn2F842L kinase may be that this allele could exhibit altered substrate recognition properties (although this is mitigated by the fact that this allele properly phosphorylates Sui2-Ser35 both *in vitro* and *in vivo*). In addition, it may be argued that our kinetic analyses of Gcn2 protein kinase activity toward eIF2α and eIF2β as independent substrates may not accurately reflect the *in vivo* conditions in which both proteins are part of the trimeric eIF2 complex.
Gcn2 kinase assays with entire elf2 complexes that carry or do not carry Sui2S52A and/or Sui3S80A mutations, may therefore more closely mimic the in vivo situation. A caveat of such assays, however, is that the Gcn2-target residues in Sui2 and Sui3 likely compete with each other for being phosphorylated by Gcn2 and that the respective phosphorylation events cannot be quantified.

Figure 4. Gcn2 engages in feedback inhibition through Gcn20-Thr94/Ser95 phosphorylation

(A) Thr94 and Ser95 in Gcn20 critically define the affinity of Gcn20 for Gcn1 in vitro. The binding affinities of recombinant His6-Gcn20, -Gcn20T94A,S95A, and -Gcn20T94E,S95E for the Alexa Fluor 488-labeled GST-EF3L domain of Gcn1 were determined by fluorescence dequenching. Three independent measurements were performed at room temperature, with SDs being <3% for each presented data point. The concentration of GST-EF3L was 50 nM. Dissociation constants (KD; n = 3; ±SD) were calculated using nonlinear asymmetric sigmoidal regressions.

(B) Thr94 and Ser95 in Gcn20 critically define the affinity of Gcn20 for Gcn1 in vivo. Cells expressing genomically WT Gcn20-HA6 (WT), Gcn20T94A,S95A-HA6 (AA), or Gcn20T94E,S95E-HA6 (EE), together with Gcn1-myc9, were grown exponentially (EXP) and then treated for 30 min with 20 mM 3-AT. Lysates (input) and anti-HA IPs (anti-HA) were analyzed by immunoblotting using anti-HA and anti-myc antibodies.

(C and D) Mutation of Thr94 and Ser95 to Glu in Gcn20 reduces the Gcn2 F842L-mediated hyperphosphorylation of eIF2α. WT and indicated mutant strains, expressing (+) or not expressing (−) plasmid-encoded hyperactive Gcn2 F842L, were grown exponentially and analyzed for eIF2α-P levels using anti-eIF2α-pSer52 and anti-eIF2α antibodies as in Figure 2E (C and D). WT samples in (C) were run on the same gel as but not next to the other samples. Strains in (D) expressed HA6-tagged Gcn20 variants that were visualized by using anti-HA antibodies. The mean ratios of eIF2α-pSer52/eIF2α (n = 3; ± SD) in (C) were normalized to the one of Gcn2F842L-expressing WT cells (set to 100%).

(E) Mutation of Thr94 and Ser95 to Glu in Gcn20 partially suppresses the growth inhibition imposed by the expression of hyperactive Gcn2 F842L. Exponentially growing strains (as in C) were spotted (10-fold serial dilutions) and grown for 3 days at 30°C on SD-Ura plates.

(F) Polysome profiles of exponentially growing WT and gcn20T94E,S95E mutant strains expressing plasmid-encoded hyperactive Gcn2 F842L. For comparison, the data of the WT control expressing Gcn2 F842L (Figure 3H; red) are shown again. The unpaired Student’s t test was used to determine the significant difference between Gcn2 F842L-expressing WT (red) and gcn20T94E,S95E cells (blue). For details, see Figure 2F.

(G) Gcn2-mediated phosphorylation of Gcn20 is part of an inhibitory feedback mechanism. The Gcn1-Gcn20 heterodimer stimulates the activation of Gcn2 by uncharged tRNAs as proposed earlier; the respective model including the domain nomenclature and representation of Gcn2 as a dimer was adapted from Hinnebusch (2005). In amino acid-replete conditions (+AA), the levels of uncharged tRNAs, and hence Gcn2 activity, are low. This favors hypophosphorylation of Gcn20 and Gcn1-Gcn20 heterodimer formation, which primes Gcn2 to optimally sense uncharged tRNAs. Upon amino acid starvation (−AA), accumulating uncharged tRNAs activate Gcn2 (a), which phosphorylates Thr94/Ser95 in Gcn20 (red circled “P”) to release it from Gcn1 (b), and thereby reduce the Gcn1-Gcn20-mediated stimulation of Gcn2 by uncharged tRNAs (c). Notably, the proposed feedback mechanism possibly also operates at a very low level when cells are growing in the presence of amino acids. The dashed lines indicate reduced function. EF3L, eukaryotic elongation translation factor 3-like domain; EF3L ABCs, EF3-related ATP-binding cassettes; HisRS, histidyl-tRNA synthase-related tRNA-binding domain; PK, protein kinase domain; A–E, subdomains of Gcn1.
individually on WT elf2 because both proteins exactly co-migrate on SDS gels.

Finally, our genetic, biochemical, and physiological experiments indicate that cells expressing Sui3GR8A and Gcn20744E/S95E maintain higher TC levels under conditions in which Gcn2 efficiently phosphorylates elf2x (e.g., nutrient starvation, rapamycin treatment). These differences in TC levels, however, are likely close to the detection limit beyond which polysome profile analyses can discriminate global translation initiation defects with statistical significance. Because the much more sensitive GCN4-LacZ assay was able to delineate the effects of the Sui3GR8A and Gcn20744E/S95E alleles, it will be interesting to characterize the translatome (using polysome and/or ribosome profiling) of respective mutants to explore the possibility that Sui3GR8A and Gcn20744E/S95E may, rather than globally controlling translation initiation, specifically affect the expression of a subset of growth-related mRNAs.

**STAR METHODS**

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**SUPPLEMENTAL INFORMATION**

Supplemental information can be found online at https://doi.org/10.1016/j.molcel.2021.02.037.

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**AUTHOR CONTRIBUTIONS**


**DECLARATION OF INTERESTS**

The authors declare no competing interests.

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**REFERENCES**


# STAR METHODS

## KEY RESOURCES TABLE

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### Experimental models: organisms/strains

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**YL516**
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**MJ5682**
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**LD5799**
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**HQY346**
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**GMGO0005**
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**LD5946**
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**LD6400**
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**LD6035**
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**LD6047**
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**LD6274**
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**LD6036**
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**LD6279**
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(Continued on next page)
RESOURCE AVAILABILITY

Lead contact
Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Claudio De Virgilio (Claudio.DeVirgilio@unifr.ch).

Materials availability
All unique/stable reagents generated in this study are available from the Lead Contact.
Data and code availability
The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PRIDE Archive: PXD021109 (Perez-Riverol et al., 2019). Source data for gel images and graphs can be found in Mendeleev Data:
https://doi.org/10.17632/g6557yvmf5.1

EXPERIMENTAL MODEL AND SUBJECT DETAILS
Saccharomyces cerevisiae strains used in this study are listed in the Key resources table. They were grown as described in Method details below. Recombinant proteins were expressed in Escherichia coli (DE3) and cloning procedures were carried out in E. coli DH5α.

METHOD DETAILS

Yeast strains, plasmids, and growth conditions
Saccharomyces cerevisiae strains and plasmids are listed in Key resources table. CRISPR-Cas9 genome editing was performed as described (Generoso et al., 2016). Gene deletion and gene tagging were performed using the pFA6a system-based PCR-toolbox (Janke et al., 2004). Plasmids used for recombinant Suí2, Suí3, and Gcn20 production were codon-optimized for expression in E. coli and purchased from GenScript. Plasmid mutagenesis was performed using the QuikChange Multi Site-Directed Mutagenesis Kit (Agilent). Unless otherwise stated, yeast strains were grown to mid-log phase in SD medium (0.17% yeast nitrogen base, 0.5% ammonium sulfate and 2% glucose). For in vivo SILAC experiments, yeast strains were grown in synthetic dextrose complete medium (or in SD medium lacking histidine and uracil in case of leucine starvation experiments) containing either non-labeled or labeled lysine and arginine variants: “Heavy” L-arginine-13C6-15N4 (Arg10) and L-lysine-13C6-15N2 (Lys8), or “medium” L-arginine-13C6 (Arg6) and L-lysine-2H4 (Lys4) amino acids (Sigma-Aldrich) were used as labels. Cells were treated or not with 200 ng mL−1 rapamycin for 30 min, or starved for leucine for 1 h. Note that for leucine starvation experiments, the cells are auxotrophic for leucine.

MS sample preparation, phosphopeptide enrichment, and LC-MS/MS analyses
MS samples and LC-MS/MS analyses were performed as described in Hu et al. (2019). Briefly, yeast strains were grown in synthetic dextrose complete medium containing either non-labeled or labeled lysine and arginine variants (see “Yeast strains, plasmids, and growth conditions”). Dried TCA-treated cell pellets (100 mg) of each labeling were mixed, proteins extracted in 8 M urea, and digested by Lys-C (Lysyl Endopeptidase, WAKO) for 4 h at room temperature. The concentration of urea was diluted to 1 M before overnight trypsin digestion (Promega). The second day, peptides were purified by SPE using HR-X columns in combination with C18 cartridges (Macherey-Nagel), eluates were frozen in liquid nitrogen and lyophilized overnight. For one biological replicate of leucine starvation, dimethyl labeling was used instead of SILAC labeling and differentially labeled peptides were mixed (Boersema et al., 2009). The third day, peptides were fractionated by HplH reversed phase chromatography, fractions were acidified, frozen in liquid nitrogen, and lyophilized overnight. The fourth day, the dry peptides were suspended in 200 μl 80% acetonitrile with 1% TFA for phosphopeptide enrichment. For both in vitro and in vivo experiments, phosphopeptides were enriched by TiO2 beads (QL Sciences). The tip flow-through was stored at −80°C for non-phosphopeptide analysis. LC-MS/MS measurements were performed on a QExactive (QE) Plus (peptides) and HF-X (phosphopeptides) mass spectrometer coupled to an EasyLC 1000 and EasyLC 1200 nano-flow-HPLC, respectively (all Thermo Scientific). Peptides were fractionated on a fused silica HPLC-column tip (I.D. 75 μm, New Objective, self-packed with ReproSil-Pur 120 C18-AQ, 1.9 μm [Dr. Maisch]) to a length of 20 cm) using a gradient of A (0.1% formic acid in water) and B (0.1% formic acid in 80% acetonitrile in water). Mass spectrometers were operated in the data-dependent mode; after each MS scan (mass range m/z = 370 – 1750; resolution: 70,000 for QE Plus and 120,000 for HF-X) a maximum of ten, or twelve MS/MS scans were performed using a normalized collision energy of 25%, a target value of 1’000 (QE Plus) or 5’000 (HF-X), and a resolution of 17’500 for QE Plus and 30’000 for HF-X. MS raw files were analyzed using MaxQuant (version 1.6.2.10) (Cox and Mann, 2008) using a UniProt full-length S. cerevisiae database (March, 2016) and common contaminants, such as keratins and enzymes used for in-gel digestion, as reference. Carbamidomethylcysteine was set as fixed modification and protein amino-terminal acetylation, serine-, threonine- and tyrosine-phosphorylation, and oxidation of methionine were set as variable modifications. The MS/MS tolerance was set to 20 ppm and three missed cleavages were allowed using trypsin/P as enzyme specificity. Peptide, site, and protein FDR based on a forward-reverse database were set to 0.01, minimum peptide length was set to 7, the minimum score for modified peptides was 40, and minimum number of peptides for identification of proteins was set to one, which must be unique. MaxQuant results were analyzed using Perseus (Tyanova et al., 2016).Motif Analysis tool (NIH) was used to predict Gcn2 phosphorylation site motif. MS data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD021109 (Perez-Riverol et al., 2019).

Protein purification
For Gcn2 purification, transformants of strain HQY346 bearing plasmids pDH103, pDH109 or pLD4074 were pre-cultured in synthetic dextrose medium lacking uracil and tryptophan, washed twice with sterile water, and suspended to OD600 of 0.4 in synthetic galac-
tose medium lacking uracil, tryptophan and histidine. At OD600 of 0.9, 10 mM 3-amino-1,2,4-triazole (3-AT) was added, and cells were further cultivated up to OD600 of 3.0. Cells were then harvested by filtration, washed with cold distilled water containing 1× complete EDTA-free protease inhibitor cocktail (PIC; Roche) and 1× Pefabloc (Sigma-Aldrich), and disrupted with glass beads using a Fast-Prep-24TM (MP Biomedicals) in binding buffer (100 mM sodium phosphate [pH 8.0], 500 mM NaCl, 0.1% Triton X-100, 1× PIC, and 1× Pefabloc). The lysates were then clarified by centrifugation (3’220 × g for 5 min at 4°C) and Gcn2 was purified using anti-FLAG M2 magnetic beads (Sigma-Aldrich). After 3 h of binding at 4°C, the beads were washed with binding buffer three times, resuspended in 20 mM Tris-HCl (pH 8), 50 mM NaCl, 1 mM DTT, 1× PIC, and 1× Pefabloc, and used in the in vitro kinase assay. The polyhistidine- and GST-fusion proteins were purified from E. coli under non-denaturing conditions using magnetic Ni-charged MagBeads or Glutathione MagBeads (GenScript), respectively, according to manufacturer’s instructions. Purified proteins were always dialyzed against the corresponding assay buffer using a Side-A-Lyzer 10K MWCO MINI dialysis device (Thermo Scientific).

**In vitro Gcn2 kinase assay**

12 μL of Gcn2, Gcn2F842L or Gcn2-kinase-dead bound to the magnetic beads were mixed with 4 μg of recombinant substrate (i.e., His5-Gcn20, His5-Sui2, or His5-Sui3; purified from E. coli) in kinase assay buffer (20 mM Tris-HCl [pH 8], 50 mM NaCl, 1 mM DTT, 1× PIC, and 1× Pefabloc). The reaction (25 μL) was started by addition of 10 μCi [γ-32P]ATP, 100 μM cold ATP and 10 mM MgCl2, and the samples were incubated for 15 min at 30°C. The reaction was stopped by adding Laemml SDS-PAGE sample buffer and incubating for 10 min at 65°C. The samples were resolved by 10% SDS-PAGE, the gel was stained with Sypro Ruby (Sigma-Aldrich), dried, and subjected to autoradiography. In parallel, the assay was performed with non-radioactive ATP (1 mM) and the samples were analyzed by MS as described (Hu et al., 2019). Kinase assays with human GCN2 (GST-tagged recombinant enzyme expressed in Sf21 insect cells; Millipore) were performed with 100 ng enzyme in assay buffer (20 mM Tris-HCl [pH 8.5], 100 mM NaCl, 200 μM EDTA, 0.1% Tween-20, and 100 μM PMFSF). The reaction (25 μL) was started by addition of 10 μCi [γ-32P]ATP, 100 μM cold ATP and 10 mM magnesium acetate; the samples were incubated for 10 min at 30°C and processed as described above. The Kₘ values were calculated with the Prism 8 software (Graphpad) using a nonlinear regression for Michaelis-Menten enzyme kinetics and interpolation from a standard curve in a confidence interval of 95%.

**Co-immunoprecipitation and immunoblot analyses**

Yeast strains were grown to mid-log phase in SD-His medium, treated, or not, with 20 mM 3-AT for 30 min, collected by filtration, and frozen in liquid nitrogen. Cells were disrupted in lysis buffer (50 mM Tris-HCl [pH 7.5], 150 mM NaCl, 50 mM NaF, 5 mM EDTA, 0.1% NP-40, 60 mM β-glycerophosphate, 1× PIC, and 1× Pefabloc) using a FastPrep-24TM, and the lysates were then clarified by centrifugation (3’220 × g for 5 min at 4°C). Co-immunoprecipitation was performed with 10 μg of total protein using PierceTM Anti-HA magnetic beads (Thermo Scientific), eIF2α subunits were detected using specific rabbit antisera (Perzlmaier et al., 2013). For HA-tag and c-Myc-tag detection, mouse anti-HA 12CA5 or anti-c-Myc 9E10 (Santa Cruz Biotechnology) antibodies were used, respectively. The beads were washed with binding buffer three times, resuspended in 1× binding buffer (3’220 × g for 5 min at 4°C), and the beads were washed with binding buffer three times, resuspended in 1× binding buffer (3’220 × g for 5 min at 4°C), and the reaction was stopped by adding Laemmli SDS-PAGE sample buffer and incubating for 5 min at 4°C. The samples were resolved by 10% SDS-PAGE, the gel was stained with Sypro Ruby (Sigma-Aldrich), dried, and subjected to autoradiography. In parallel, the assay was performed with non-radioactive ATP (1 mM) and the samples were analyzed by MS as described (Hu et al., 2019). Kinase assays with human GCN2 (GST-tagged recombinant enzyme expressed in Sf21 insect cells; Millipore) were performed with 100 ng enzyme in assay buffer (20 mM Tris-HCl [pH 8.5], 100 mM NaCl, 200 μM EDTA, 0.1% Tween-20, and 100 μM PMFSF). The reaction (25 μL) was started by addition of 10 μCi [γ-32P]ATP, 100 μM cold ATP and 10 mM magnesium acetate; the samples were incubated for 10 min at 30°C and processed as described above. The Kₘ values were calculated with the Prism 8 software (Graphpad) using a nonlinear regression for Michaelis-Menten enzyme kinetics and interpolation from a standard curve in a confidence interval of 95%.

**β-Galactosidase assays**

Yeast strains carrying p180 (GCN4-LacZ (Hinnebusch, 1985)), p367 (HIS4-AUG-LacZ) or p391 (his4-UUG-LacZ (Donahue and Cigan, 1988)) were grown in synthetic complete medium lacking uracil and histidine. GCN4 derepressing conditions were imposed by treatment with 40 mM 3-AT or 200 ng mL⁻¹ rapamycin for 4 h. Cell pellets were resuspended in Z-buffer and processed for β-galactosidase assay with the SDS/chloroform cell permeabilization method as previously described (Guarente, 1983). β-galactosidase activity was measured using 2-nitrophenyl-β-D-galactopyranoside as substrate.

**Polysome profile analysis**

Yeast cultures (200 mL) were grown in YPD at 30°C to OD600 of 0.6 and treated, or not, with 200 ng mL⁻¹ rapamycin for 30 min. For the polysome analyses with strains expressing Gcn2F842L (Figures 3H and 4F), strains were grown exponentially in SD-Ura at 30°C to OD600 of 0.6. Cycloheximide was added to a final concentration of 0.1 mg mL⁻¹, and the cultures were immediately placed on ice and shaken for 5 min. Cells were harvested by centrifugation, washed with 20 mL of ice-cold breaking buffer (10 mM Tris-HCl [pH 7.5], 100 mM NaCl, 30 mM MgCl₂, and 0.1 mg mL⁻¹ cycloheximide), and resuspended in 0.8 mL breaking buffer. Glass beads were added to about one-fourth of the total volume and vortexed 8 times for 30 s with 30 s intervals on ice. The extracts were clarified by centrifugation (12’000 × g for 10 min at 4°C). Eight A660 units were layered onto 10%–50% sucrose gradients containing 50 mM Tris-acetate (pH 7.5), 50 mM NH₄Cl, and 12 mM MgCl₂, which were then centrifuged at 39’000 rpm in a Sorvall TH-641 rotor at 6°C for 2 h 45 min. Sucrose gradients were analyzed using an ISCO UA-6 system with continuous monitoring at A₅₂₅₄.

**Fluorescence spectroscopy**

Fluorescence spectroscopy was used to quantify affinities of protein-protein and protein-peptide interactions by fluorescence (de) quenching. GST-EF3L (Gcn11331-1670) labeling by Alexa Fluor 488 5-TFP (Life Technologies) was performed as described.
previously (Janović et al., 2019). Measurements of EF3L interactions with Gcn20 variants were performed at room temperature in phosphate-buffered saline (pH 7.4) on a FluoroMax-4 spectrofluorometer (Horiba), monitoring fluorescence intensity at an excitation wavelength of 489 nm and an emission wavelength of 515 nm. The slit width was 10 nm and the integration time was 1 s. Peptides PTDDIAEALGELSLKKKKKTK and PTDDIAEALGEL[pSer]LKKKKKTK corresponding to Sui368-89 were N-terminally conjugated with fluorescein isothiocyanate over 6-aminohexanoic acid (GenScript), and tested for GST-Tif5 and GST-Gcd6 binding at room temperature in assay buffer (30 mM HEPES [pH = 7.5], 100 mM KCl, 0.1 mM MgCl₂, 10% glycerol, 1 mM DTT, 0.1 mM PMSF, 0.5 × PhosSTOP) on a Cytation 5 plate reader (BioTek), monitoring fluorescence intensity at an excitation wavelength of 485 nm and an emission wavelength of 528 nm. Kds were calculated by Prism 8 software (Graphpad) using nonlinear asymmetric sigmoidal regression (5PL). The experiments were performed in triplicates.

QUANTIFICATION AND STATISTICAL ANALYSIS

Statistical parameters are reported in the Figures and Figure Legends.