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Editing of misaminoacylated tRNA controls the sensitivity of amino acid stress responses in *Saccharomyces cerevisiae*

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ABSTRACT

Amino acid starvation activates the protein kinase Gcn2p, leading to changes in gene expression and translation. Gcn2p is activated by deacylated tRNA, which accumulates when tRNA aminoacylation is limited by lack of substrates or inhibition of synthesis. Pairing of amino acids and deacylated tRNAs is catalyzed by aminoacyl-tRNA synthetases, which use quality control pathways to maintain substrate specificity. Phenylalanyl-tRNA synthetase (PheRS) maintains specificity via an editing pathway that targets non-cognate Tyr-tRNA^{Phe}. While the primary role of aaRS editing is to prevent misaminoacylation, we demonstrate editing of misaminoacylated tRNA is also required for detection of amino acid starvation by Gcn2p. Ablation of PheRS editing caused accumulation of Tyr-tRNA^{Phe} (5%), but not deacylated tRNA^{Phe} during amino acid starvation, limiting Gcn2p kinase activity and suppressing Gcn4p-dependent gene expression. While the PheRS-editing ablated strain grew 50% slower and displayed a 27-fold increase in the rate of mistranslation of Phe codons as Tyr compared to wild type, the increase in mistranslation was insufficient to activate an unfolded protein stress response. These findings show that during amino acid starvation a primary role of aaRS quality control is to help the cell mount an effective stress response, independent of the role of editing in maintaining translational accuracy.

INTRODUCTION

tRNA is the most abundant non-coding RNA, comprising 4–10% of the total intracellular RNA pool (1,2). Regulation of the biosynthesis, modification, and degradation of tRNA directly impacts translation and extends the role of tRNA beyond the canonical function of decoding genetic information (3,4). Numerous defects in tRNA biogenesis and related processes, including tRNA aminoacylation, have now been linked to human disease (5,6). As primary determinants of the genetic code, aminoacyl-tRNA synthetases (aaRS) are responsible for pairing amino acids with cognate tRNAs. For example, phenylalanyl-tRNA synthetase (PheRS) is responsible for pairing phenylalanine with its cognate tRNA^{Phe} isoacceptors (7). Mispaired aminoacyl-tRNAs (aa-tRNA) are occasionally made due to recognition of non-cognate amino acids within the PheRS active site, for example when tyrosine is misacylated onto tRNA^{Phe} (Tyr-tRNA^{Phe}) (8). AaRS proofreading mechanisms have evolved to restrict misaminoacylated tRNA accumulation through hydrolysis of misactivated aminoacyl adenylates (pre-transfer editing) and hydrolysis of misaminoacylated aa-tRNA (post-transfer editing). While aa-tRNA proofreading plays a role in minimizing mistranslation, far less is known about how this conserved step in translation quality control might regulate other cellular processes (9,10).

Aminoacylation of the intracellular tRNA pool is a primary signal for cellular stress response pathways in both

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bacteria and eukaryotes. In eukaryotes, the general amino acid control (GAAC) pathway controls cellular translation as a means to regulate the transcriptional response to amino acid starvation (11–15). The GAAC indirectly monitors intracellular amino acid pools through surveillance of deacylated tRNA accumulation via the protein kinase Gcn2p. Activation of the GAAC occurs when deacylated tRNA binds to a region of Gcn2p homologous to histidyl-tRNA synthetase, thereby disrupting interdomain interactions (16,17). Activated Gcn2p phosphorylates eIF2α, which competitively inhibits the conversion of eIF2-GDP to eIF2-GTP, which in turn reduces the pool of active ternary complex (TC) available for translation initiation. Reduced levels of TC decrease global translation but increase production of Gcn4p, a transcription factor required for cellular responses to amino acid deprivation (18). In bacteria accumulation of deacylated tRNA activates the stringent response, which like the GAAC leads to changes in gene expression at the levels of both transcription and translation (19,20). When deacylated tRNA enters the A-site of the bacterial ribosome, the enzyme RelA activates the stringent response through the production of the second messenger ppGpp(p). ppGpp(p) directly affects the translational and transcriptional status of the cell in response to nutrient stress (19). In bacteria, loss of aaRS-mediated tRNA quality control limits deacylated tRNA accumulation during amino acid starvation and suppresses the stringent response (10). The loss of aaRS editing of misaminoacylated tRNAs also leads to significant activation of protein stress responses, presumably in response to increased accumulation of misfolded proteins resulting from mistranslation. While these studies identified a role for translational quality control in determining the sensitivity and specificity of nutritional stress responses, whether this function is dependent on mistranslation remained unclear. To investigate the mechanisms by which editing of misaminoacylated tRNAs regulate cellular stress responses, we used the yeast *Saccharomyces cerevisiae* to determine whether eukaryotic amino acid starvation sensing is also linked to translation quality control (10). As with the bacterial stringent response, we found that accurate monitoring of amino acid starvation by the yeast GAAC is dependent on aaRS editing translation quality control to ensure proper accumulation of deacylated tRNA species. In the absence of editing under these growth conditions, misaminoacylated tRNAs accumulated to significant levels, but the yeast protein stress responses were not activated. Taken together these data reveal a critical function for aaRS-editing in stress responses that is independent of their role in preventing mistranslation.

MATERIALS AND METHODS

Genetic techniques

Construction of strains NR1 and NR2 was described previously (21). Amino acid prototrophic strains KM03 and KM04 were constructed by derivatization of NR1 and NR2, respectively, through homologous recombination of WT *HIS3* and *LEU2* alleles amplified from strain S288c by PCR, as described previously. Deletion of *GCN2* from strains KM03 and KM04 by homologous recombination of a *GCN2* targeted KanMX4 cassette yielded yeast strains KM17 and KM18, respectively. Likewise, yeast strains KM19 and KM20 were created through derivatization of KM03 and KM04 by replacement of the WT *GCN4* allele with a KanMX4 cassette (Supplementary Table S2).

Growth analysis and complementation

Growth of all *Saccharomyces cerevisiae* strains was conducted as described previously (22). Synthetic defined minimal media (SDMM) was comprised of Difco yeast nitrogen base without amino acids, 2% glucose, 0.002% adenine, 0.002% uracil, 0.002% L-histidine and 0.01% L-leucine. Tyrosine stress media (SDMM + Phe:Tyr) was made with SDMM and varying concentrations of Phe:Tyr where [Phe] was kept at 0.003 mM and [Tyr] varied from 0.003–1.2 mM. Synthetic complete media (SC) was made using drop out base powder (DOB) and SC supplement (Sunrise Science). For all growth assays, *S. cerevisiae* strains NR1, NR2 and derivatives were streaked on YPDA and grown at 30°C for 72 h. Single colonies were suspended in sterile water and used to inoculate 250 μl of media in a microtiter plate to a final OD_{600} of 0.01. Growth was monitored using an xMark microplate absorbance spectrophotometer (Bio-Rad Laboratories, Hercules, CA, USA) by measuring the absorbance at 600 nm every five minutes for 24–48 h. Complementation of *frs1-1* strains was achieved through transformation of plasmid pFL36 containing a WT copy of *FRS1* and a *URA3* selection cassette. Growth was monitored as described above.

Spot assay

Yeast cultures were prepared by streaking strains from frozen stocks onto YPD agar plates. After incubating the cells at 30°C for 48 h, single colonies were resuspended in autoclaved H_2O to a final OD_{600} of 1.0. Serial dilutions (10^{-1} to 10^{-4}) were prepared by mixing 100 μl of culture into 900 μl sterile H_2O. From each dilution, 8 μl was spotted in sequence onto solid agar plates with varying composition depending upon the strain and experimental design. Plates were incubated at 30°C for 48 h and scanned for growth comparison.

β-galactosidase assay

Cultures were inoculated to an OD_{600} of 0.01 in various media. Culture (1 ml) at an OD_{600} of 0.8–1.0 was harvested by centrifugation and resuspended in 1 ml of Z-Buffer (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgSO_4$ and 0.27% mercaptoethanol). Cells were permeabilized using 20 μl 0.1% SDS and 40 μl $CHCl_3$, vortexed for 15 s and incubated at 30°C for 10 min. Permeabilized cell suspension (125 μl) was removed from the aqueous layer into a 96-well plate, diluted with an equal volume of Z-buffer, and the absorbance at 600 nm was determined. Subsequently, 200 μl of the same cell suspension was removed from the aqueous layer into a 96 well plate and mixed with 50 μl of 5 mg/ml CPRG substrate to a final concentration of 1 mg/ml. The absorbance at 580 nm was recorded at regular intervals and the linear rate was determined. Enzyme activity was determined from linear rates normalized to absorbance at

600 nm. All absorbance measurements were recorded using an xMark microplate absorbance spectrophotometer (Bio-Rad Laboratories, Hercules, CA, USA).

Transcription analyses

For Northern blot analysis strains NR1 and NR2 were inoculated into 50 ml liquid YPDA, minimal media (MM) + Phe:Tyr 1:1 (0.003 mM Phe, 0.003 mM Tyr), or minimal media (MM) + Phe:Tyr 1:50 (0.003 mM Phe, 0.15 mM Tyr) and grown to an OD_{600} of 0.8. Cells were harvested by centrifugation, washed with 50 ml ddH_2O, frozen, and stored at –80°C overnight for RNA extraction. The unfolded protein response was induced by treating cultures at an OD_{600} of 0.4 with tunicamycin (10 μg/ml), grown to OD_{600} of 0.8 and processed as above. Total cellular RNA was extracted using hot phenol. Northern blot analysis was carried out using the NorthernMax–Gly Kit (Ambion/Life Technologies, San Francisco, CA, USA) according to the manufacturer's instructions. Agarose gels were loaded with 5 μg of total RNA for each sample. A single stranded DNA probe (5′-CAAACAAATTGTTGTTGTCTACGGCAGGTAG-3′) complementary to base pairs 529–559 of the *HAC1* transcript was radiolabeled with [γ-^{32}P]-ATP by T4 polynucleotide kinase and utilized for hybridization.

For RT-qPCR analysis cells were inoculated to an OD_{600} of 0.1 in 5 ml tubes, then incubated at 30°C with shaking. Cells were harvested at an OD_{600} of 0.8–1.0 by centrifugation and resuspended in 1 ml of TriZoL reagent (Life Technologies, San Francisco, CA, USA). Total RNA was extracted per manufacturer's instructions. RNA quantity and integrity was determined using a Bioanalyzer 2000 (Agilent Technologies, Santa Clara, CA, USA). cDNA was synthesized from 500 ng of total RNA using SSIV reverse transcriptase (Life Technologies, San Fransisco, CA, USA) and random hexamer primer (ThermoFisher, Waltham, MA, USA) per manufacturer's instructions. qPCR was conducted using SYBR iQ master mix (Bio-Rad Laboratories, Hercules, CA, USA) and primer sets specific for each target (Supplementary Table S3). Data was collected using a CFX96 thermal cycler (Bio-Rad Laboratories, Hercules, CA, USA) and analyzed according to the Pfaffl method (23).

Cell size measurement

Synchronous early G1 populations of cells from strains NR1 and NR2 were harvested by centrifugal elutriation, as described previously (24). Briefly, cells were grown in YPD or SDMM stress medium to a cell density of 1–5 × 10^7 cells/ml and loaded onto an elutriator chamber at a pump speed of 35 ml/min spinning at 3200 rpm (Beckman J-6M/E centrifuge). Centrifugation steps were all completed at 25°C. The elutriated small daughter cells were collected at 2400 rpm centrifuge speed and 40 ml/min pump speed, in tubes kept on ice. The elutriated cells were recovered by centrifugation and re-suspended in fresh pre-warmed medium, at a cell density of ∼1 × 10^7 cells/ml. The elutriated cultures were then placed in a 30°C incubator and every 20 min we recorded their budding index and measured their cell size with a Beckman Z2 channelyzer. We recorded the geometric mean of the cell size of the population, using the Accucomp software package of the instrument. Birth and mean cell size were determined from asynchronous cell populations, as described previously (25).

Immunoblotting

Strains were cultured in 50 ml of SDMM with or without Phe and Tyr and harvested at an OD_{600} of 0.8–1.0. Cells were harvested by centrifugation at 4°C, resuspended in modified RIPA buffer (10 mM Tris–HCl pH 7.5, 100 mM NaCl, 1 mM EDTA, 1 mM EGTA, 0.1% SDS, 0.5% sodium deoxycholate, 1% triton-X 100, 10% glycerol), supplemented with Complete Mini protease inhibitor cocktail and PhosSTOP phosphatase inhibitor cocktail (Roche, Basel, Switzerland). Cells were lysed by vortexing with glass beads followed by centrifugation to clarify the lysate. Total protein concentration was assessed using the Bradford method, separated by electrophoresis using a 10% SDS-polyacrylamide gel, and transferred to a PVDF membrane per manufacturer's recommendations (Abcam ab4837). Immunoblot analysis was conducted using a 1:1000 dilution of a polyclonal antibody that recognizes the phosphorylated eIF2α at Ser-51 (Abcam, Cambridge, UK), followed by hybridization with 1:20 000 dilution of donkey α-rabbit HRP conjugate antibody (GE Healthcare, Waukesha, WI, USA). Protein was quantified using chemiluminescent substrate. Membranes were stripped and re-probed for GAPDH to identify variations in sample loading.

Quantification of Aminoacyl-tRNA

Strains were cultured in 50 ml of SDMM with or without Phe and Tyr and harvested at an OD_{600} of 0.8–1.0. RNA quantity and integrity was determined using a Bioanalyzer 2000 (Agilent Technologies, Santa Clara, CA, USA). Samples were run on a 12% denaturing urea-acid acrylamide gel (8 M urea, 12% acrylamide 19:1, 100 mM NaOAc pH 5.2, 1% APS) at a constant 13 watts for 24 h at 4°C. RNA was transferred onto a nitrocellulose membrane and processed as described previously (10). The membrane was probed with ^{32}P end-labeled oligo (5′-GATCGAACACAGGACCTCCAGAT-3′) specific for $tRNA^{Phe}$. Post hybridization, a phosphor screen was exposed to the membrane for 24–72 h and scanned using a Typhoon FLA 7000 (GE Healthcare, Waukesha, WI, USA).

Isoacceptor specific aminoacylation profiling (ISAP)

Total tRNA purification was conducted as described above. Total tRNA was then incubated with 1 μM 5′ biotinylated probe in 500 μl of 2× SSC, pH 4.8 at 50°C for 1 h. After hybridization, the tube was allowed to cool to room temperature. Streptavidin paramagnetic beads (SA-PMPs) were re-suspended in storage buffer and captured using a magnetic pipet. SA-PMPs were washed three times with 300 μl of 2× SSC, pH 4.8 and re-suspended in 100 μl of 2× SSC, pH 4.8. The annealed oligonucleotides were captured by adding the 500 μl RNA-Probe mixture to 100 μl of pre-washed SA-PMPs, incubated at RT for 10 min and gently mixed by inverting the tube every 1–2 min. The SA-PMPs were then

captured using a magnetic pipet and washed six times with 300 μl of 2× SSC, pH 4.8. Aa-tRNA^{Phe} was deacylated by incubating the SA-PMPs in 20 mM ammonium formate, pH, 10 at 37°C for 1 h. Following deacylation, SA-PMPs were removed using a magnetic pipet. The supernatant was dried down and amino acids were quantified via mass spectrometry, as described previously (26).

Mass spectrometry mistranslation reporter

Yeast cultures were grown at 30°C with shaking at 250 rpm until mid-log phase, quenched on ice and pelleted at 2000 × g (15 min at 4°C). The supernatant was discarded and the cell pellets were frozen at –80°C to assist with subsequent protein extraction. Frozen cell pellets were thawed and incubated with 300 U/ml of zymolyase for 20 min at RT. Lysis buffer consisting of 50 mM Tris–HCl (pH 7.4, 23°C), 500 mM NaCl, 0.5 mM EGTA, 1mM DTT, 10% glycerol, 50 mM NaF and 1 mM Na_3O_4V was added to the digested cells, along with sterile 0.1 mm acid washed glass beads. Cell suspensions were vortexed six times, 30 s per round. Samples were briefly sonicated and the supernatant was removed after two rounds of centrifugation at 14 000 × g for 20 min. The remaining pellet was re-extracted and resulting fractions were combined. Cell free extracts were applied to Ni-NTA metal affinity resin and purified according to the manufacturer's instructions. Wash buffers contained 50 mM Tris pH 7.5, 500 mM NaCl, 0.5 mM EGTA, 1 mM DTT, 50 mM NaF, 1 mM Na_3VO_4 and increasing concentrations of imidazole 20, 40 and 60 mM, sequentially. Proteins were eluted with buffer containing 250 mM imidazole. Eluted protein was subjected to four rounds of buffer exchange (20 mM Tris pH 8.0 and 100 mM NaCl) and concentrated using a 3 kDa molecular weight cutoff spin filter (Amicon). SDS-PAGE followed by staining with Coomassie blue revealed >90% purity. Protein concentration was determined by absorbance at 280 nm using a nanodrop spectrophotometer. Samples were frozen and stored at –80°C. An aliquot corresponding to 20 μg GFP was transferred into a 1.5-ml PCR tube and the composition of the sample was adjusted using stock solutions consisting of 100 mM Tris–HCl buffer pH 8.0, 100 mM DTT, 100 mM EDTA, 5% ALS-110 (Protea) and water. The final sample composition was 20 μg GFP dissolved in 40 μl 10 mM Tris–HCl, pH 8.0 (23°C), 0.5% ALS-110, 10 mM DTT and 1 mM EDTA. Disulfides were reduced with DTT for 35 min at 55°C in a water bath. The reaction was quenched on ice and 16 μl of 60 mM IAA was added for alkylation of thiols. The reaction proceeded for 30 min at room temperature and in the dark. Excess IAA was quenched with 14 μl of 25 mM DTT. The digest was then diluted with 40 μl of 1M Tris–HCl, pH 8.0 and 310 μl of 70 mM Tris–HCl, pH 8.0 containing 2 mM $CaCl_2$. Sequencing grade trypsin prepared at 0.5 μg/μl was added to obtain a trypsin/protein ratio of 1:15 by weight and protein was digested for 16 h at 37°C. The digest was quenched with 64 μl of a 20% TFA solution. Cleavage of the acid cleavable detergent was performed for 15 min at room temperature, and peptides were desalted on a C_{18} UltraMicroSpin Column (The Nest Group Inc., Southborough, MA, USA). Peptides were dried in a vacuum centrifuge at RT. Dried samples were dissolved in 2.6 μl 1-propanol, 2 μl 70% FA and 15.4 μl 0.5% acetic acid and stored at −80°C until further analysis. LC–MS/MS was performed on an Orbitrap Velos as described previously (27) with the following changes. Nano liquid chromatography was performed with a vented split setup consisting of a fused silica trap column (30 mm × 150 μm ID) fitted with a Kasil frit following a protocol by Link (28). The trap column was connected to a metal nano T that was connected to an external switching valve. The electrospray voltage, typically 1500–1800 V was applied to the metal T using an alligator clip. The trap column contained Reprosil-Pur resin with 120 Å pore size and 3 μm particle size (Dr Maisch GmBH, Ammerbuch Germany). Trapping was performed for 3.75 min at a flow rate of 4 μl/min with 5% eluent B (defined below). The analytical column was a 75 μm ID PicoFrit column (New Objectives, Woburn, MA, USA) packed with 20 cm of 1.9 μm diameter Reprosil-Pur 120 C18-AQ C_{18} particles (Dr Maisch GmBH, Ammerbuch Germany) using methanol as the packing solvent. Peptides were separated at a flow rate of 300 nl/min using a 90 min gradient with 0.1% FA (Eluent A) and 0.1% FA in acetonitrile (Eluent B). The linear gradient was as follows (min/%B): 0.0/5.0, 0.1/5.0, 45.0/25.0, 65.0/50.0, 66.0/95.0, 71.0/95.0 72.0/5.0, 90.0/5.0. An estimated 100 ng of the peptide digest dissolved in 2% Eluent B was injected for analysis. Database searching was performed with MaxQuant v 1.5.3.30 against a custom database of GFP X16 containing all natural AAs at position X16, and a *S. cerevisiae* protein database (Uniprot, strain AWRI1631, 5450 sequences). Forward and decoy database searches were carried out using full trypsin specificity with up to three missed cleavages and using a mass tolerance of 30 ppm for the precursor and 0.1 Da for fragment ions, respectively.

RESULTS

Loss of PheRS editing activity leads to accumulation of mis-aminoacylated tRNA^{Phe}

Previous studies showed that a D243A amino acid replacement in the β-subunit of yeast cytoplasmic PheRS leads to loss of editing activity against Tyr-tRNA^{Phe} (29). The corresponding mutation was introduced into the WT allele (*FRS1*) to generate a strain defective in PheRS editing (*frs1-1*; (21)), which was used to investigate the effects of QC on tRNA aminoacylation under stress conditions. When grown in the presence of low phenylalanine / high tyrosine (1:400, Phe:Tyr) the QC deficient strain showed an increase in aa-tRNA^{Phe} levels compared to wild type. Approximately 70% of total cellular tRNA^{Phe} was aminoacylated for WT and QC deficient strains grown in minimal medium. When Tyr was added to the medium, levels of aminoacylated tRNA^{Phe} in the WT strain remained at 70%, while levels in the QC deficient strain rose to ∼75%, similar to WT aminoacylation levels on rich medium (Figure 1A). To determine if the increase in aa-tRNA^{Phe} in the QC defective strain was due to Tyr-tRNA^{Phe} accumulation, tRNA isoacceptor specific aminoacylation profiling (ISAP) was used to quantify amino acids attached to tRNA^{Phe} *in vivo* (26). tRNA^{Phe} species were isolated from total aa-tRNA extracted from both WT and QC deficient strains grown in minimal medium with or without high Tyr stress. Isolated tRNA^{Phe} was washed until free of detectable unbound

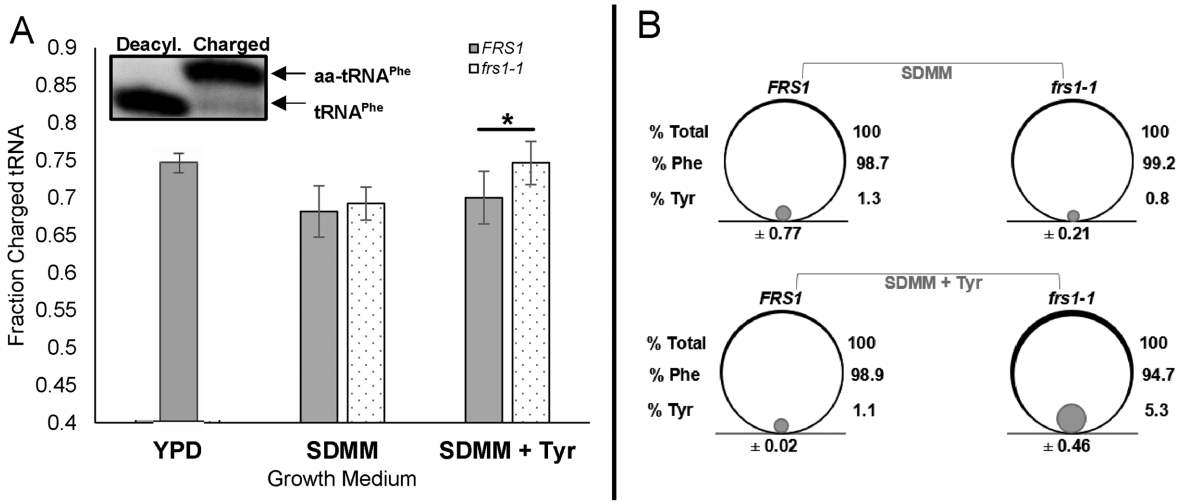

Figure 1. Characterization of aminoacyl-tRNA^{Phe}. (**A**) Levels of aminoacylated tRNA^{Phe} were measured from KM03 (*FRS1*) and KM04 (*frs1-1*) cells grown in minimal medium (SDMM) or SDMM supplemented with 1.2 mM tyrosine (SDMM + Tyr) where indicated. Total RNA was extracted under acidic conditions and electrophoretically separated from deacylated tRNA^{Phe} on an acid-urea-polyacrylamide gel. Northern blot analysis was conducted using a 32^{P}-end labeled probe specific for tRNA^{Phe}. Densitometry analysis was conducted on images using Image J. Error bars represent the S.D. determined from at least nine independent experiments. $P < 0.01$. (**B**) WT and QC deficient cells were grown in minimal (SDMM) or high Tyr (1:400) medium. aa-tRNA was extracted and specific tRNA^{Phe} isoacceptors were isolated through hybridization with a 5′ biotinylated oligo specific for tRNA^{Phe}. Amino acids were removed from isolated tRNA and identified by mass spectrometry from three separate experiments.

amino acids and subsequently deacylated. Free amino acids from the deacylated fraction were identified and quantified by mass spectrometry. tRNA^{Phe} isolated from the WT strain grown in both minimal medium and minimal medium supplemented with Tyr showed ∼1.0% misacylation with Tyr, consistent with the efficiency of PheRS aa-tRNA editing activity determined *in vitro* (22). Similar to WT, samples from the QC deficient strain grown in minimal medium showed ∼1.0% misacylation, while misacylation of tRNA^{Phe} with Tyr in the QC deficient strain grown in high Tyr medium increased to ∼5.0% (Figure 1B). These data indicate that in the absence of PheRS editing there is an increase in the ratio of aminoacylated to deacylated tRNA^{Phe} as a result of increased cellular levels of misaminoacylated Tyr-tRNA^{Phe}.

PheRS editing activity is required for optimal growth during amino acid stress

The growth of WT (*FRS1*) and PheRS QC deficient (*frs1-1*) yeast strains was characterized under different growth conditions at both population and single cell resolution (21,22). In response to high levels of extracellular Tyr and Phe limitation (Tyr:Phe, 400:1), the PheRS QC deficient strain grew ∼50% slower than WT (Figure 2A, Supplementary Table S1). Complementation of the QC deficient strain with a plasmid harboring a WT copy of *FRS1* partially restored growth in high tyrosine media (Figure 2B), while the addition of an equimolar amount of cognate Phe to the media restored growth to near WT levels (Figure 2A). Rescue of growth by WT *FRS1* complementation or exogenous Phe supplementation suggests that the growth deficiency results from loss of the Tyr-tRNA^{Phe} editing activity of PheRS.

To further characterize the PheRS QC deficient strain, flow cytometry was used to measure cell size for unsynchronized populations by monitoring the distribution of front scatter signal (FSC), a measure of particle size. The QC deficient strain showed a substantial increase in average population size when compared to the WT strain grown in media supplemented with high levels of Tyr. To accurately determine the population size parameters, WT and QC deficient strains were analyzed using a channelyzer. WT and QC deficient cells have similar size distributions in YPD, but significantly different cell size distributions when grown asynchronously in high Tyr medium (Table 1). On high Tyr, the average size of WT cells was 57 fl, while the average size of the QC deficient strain increased to 73 fl (Figure 3). To determine if these differences in size were indicative of changes to the kinetics of cell cycle progression for *frs1-1* cells, the critical size that cells must reach in order to commit to a new round of cell division was determined using synchronous cultures. On high Tyr, WT cell critical size was reduced to 43 fl, while the critical size of the QC deficient strain remained relatively consistent at 60 fl across all growth conditions (Table 1). Decrease in critical size is commonly observed in cells that undergo nutritional stress and often results in cell cycle arrest in the G1 phase (30). The observation that the QC deficient strain did not decrease critical size in response to high Tyr growth conditions suggests that its reduced growth rate may result from misregulation of a cellular response to nutritional stress.

Deficiency in aa-tRNA editing suppresses activation of the GAAC

One of the primary responses to nutritional stress in yeast is the GAAC, which mediates cellular reprogramming of gene expression in response to fluctuations in amino acid availability, with deacylated tRNA serving as the primary trigger (18). Reliance on changes in the ratio of aminoacylated to deacylated tRNA as a primary sensor suggests that aa-

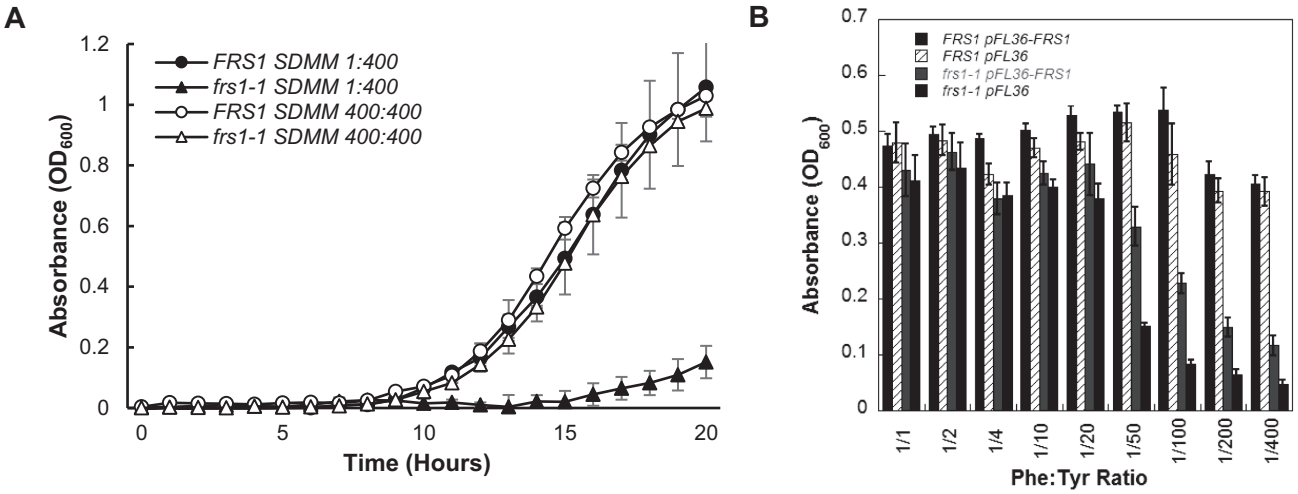

Figure 2. Growth and complementation of WT (*FRS1*) and PheRS editing deficient (*frs1-1*) strains. Complementation of chromosomal NR1 (*FRS1*) and NR2 (*frs1-1*) strains with pFL36-*FRS1* or pFL36. (**A**) Cells were grown in 150 μl of minimal media plus 0.003 mM Phe and a variable concentration of Tyr. Data points are an average of at least three independent experiments, with errors bars representing 1 SD. (**B**) Synthetic defined minimal medium (SDMM) was supplemented with 1:400 or 400:400, Phe:Tyr (1 = 0.003 mM and 400 = 1.2 mM) was inoculated with either *FRS1* (Circles) or *frs1-1* (triangles) cells to a starting OD600 of 0.01. Growth was monitored by measuring the absorbance at 600nm using a microplate spectrophotometer. Each value represents three biological replicates.

Table 1. Cell size parameters shift in response to high Tyr stress

YPD	Birth size	Mean size	Critical size
FRS1	31.09 ± 0.75	62.33 ± 1.65	57.01 ± 3.44
frs1-1	32.75 ± 1.71	67.01 ± 2.00	62.03 ± 0.12
SDMM + Tyr	Birth size	Mean size	Critical size
FRS1	25.69 ± 1.03	56.94 ± 1.04	43.47 ± 0.86
frs1-1	37.03 ± 1.41	72.91 ± 1.89	65.16 ± 0.08

FRS1 and *frs1-1* cells were synchronized by centrifugal elutriation and resuspended in rich medium (YPD) or minimal medium (SDMM) supplemented with 1.2mM Tyr. Size parameters are volume measurements obtained using a Coulter Counter Z2. Error represents 1 S.D. from three independent experiments.

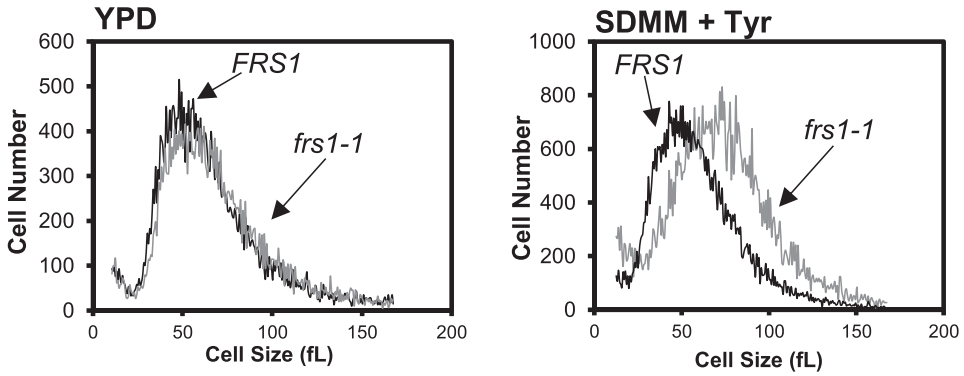

Figure 3. Single cell analysis of cell size distribution. Strains NR1 (*FRS1*) and NR2 (*frs1-1*) cells were synchronized by centrifugal elutriation and resuspended in rich medium (YPD) or minimal medium (SDMM) supplemented with 1.2 mM Tyr. Size parameters are volume measurements obtained using a Coulter Counter Z2. Error represents 1 S.D. from three independent experiments.

tRNA proofreading may play a key role in the selectivity and sensitivity of the GAAC response. In the absence of QC, changes in the extracellular Phe:Tyr ratio led to increased Tyr-tRNA^{Phe} levels (see above). To investigate whether increased tRNA misacylation impacts the ability of the cell to accurately respond to amino acid limitation, activation of the GAAC was monitored in response to amino acid stress in wildtype and PheRS QC deficient strains. The GAAC is initiated by the binding of deacylated tRNA to Gcn2p, which activates its kinase activity leading to the phosphorylation of eIF2α. Consistent with a reduction in deacylated tRNA accumulation under amino acid stress, the amount of phosphorylated eIF2α decreased ∼4-fold in the QC deficient strain compared to WT when challenged with high Tyr stress (Figure 4A). Whether the observed reduction in eIF2α phosphorylation was sufficient to significantly re-

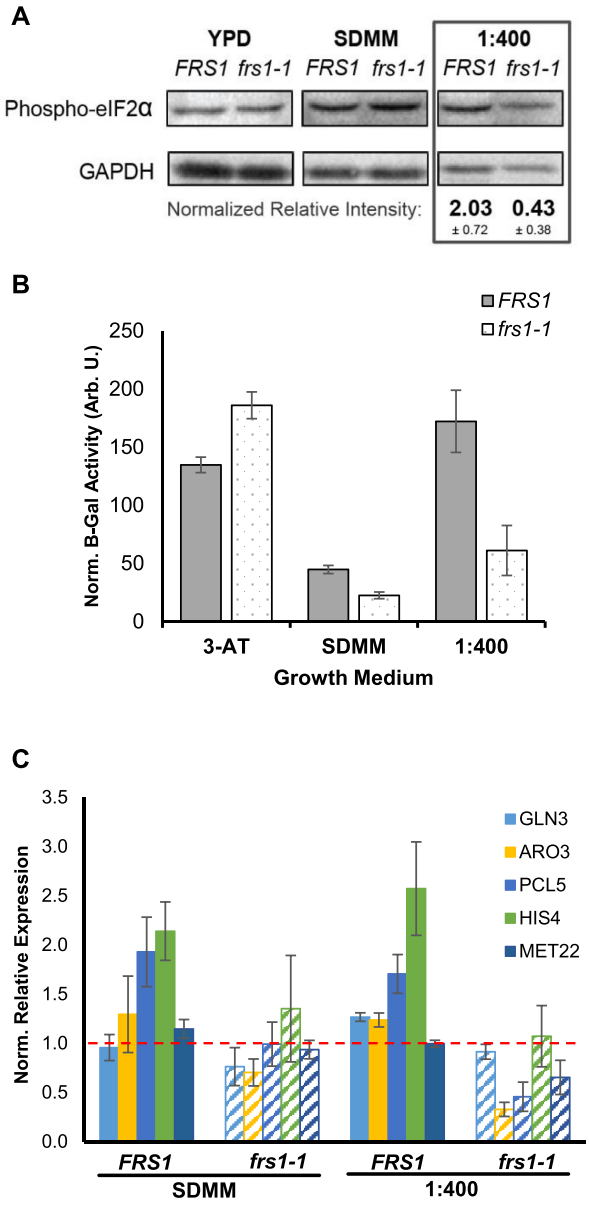

Figure 4. Activation of GAAC and regulation of downstream targets. (**A**) Total protein was extracted from KM03 (*FRS1*) or KM04 (*frs1-1*) cells grown in the indicated medium. Protein isolate (30 μg) was resolved electrophoretically on an SDS-polyacrylamide gel and analyzed by immunoblot using an antibody specific for the phosphorylated form of eIF2α. Loading and band quantification was verified by immunoblot against GAPDH. (**B**) KM03 (*FRS1*) and KM04 (*frs1-1*) cells harboring plasmid p180 (uORF-GCN4:LacZ) were grown in the indicated medium. Beta-galactosidase activity was determined using CPRG chromogenic substrate by monitoring the OD_{580} over time. Beta-galactosidase activity was measured as the rate of substrate turnover and then normalizing internally to each independent sample. Error bars represent 1 S.D. determined from three independent experiments. (**C**) Transcript abundance for targets activated by Gcn4p was analyzed by qRT-PCR. cDNA libraries were prepared from 500 ng total RNA extracted from KM03 (*FRS1*) or KM04 (*frs1-1*) cells grown to an OD_{600} of 0.8–1.0 in minimal medium (SDMM) or SDMM supplemented with 1:400, Phe:Tyr (1 = 0.003 mM). cDNA was analyzed by qPCR using SYBR Green I. Samples were normalized internally to levels of ACT1 and reported relative to WT expression in rich medium (YPD). Error bars represent S.D. from at least three independent experiments.

duce translation of *GCN4* was assessed using a *GCN4:LacZ* reporter plasmid harboring the complete uORF regulatory region found in the 5′ UTR of the native GCN4 mRNA (31,32). Translation of *GCN4* mRNA, as measured by the reporter, was significantly reduced (∼3-fold) in the editing deficient strain when subjected to Tyr stress, while activity in the WT (*FRS1*) strain more closely mirrored reporter activity observed in cells grown in the presence of 3-amino-1,2,4-triazole (3-AT), a chemical activator of the GAAC (Figure 4B). To confirm that Gcn4p activity was reduced in *frs1-1* cells during Tyr stress, transcript abundance of select downstream targets of Gcn4p were measured using RT-qPCR. The editing deficient strain grown in high Tyr media has significantly lower steady state levels of several transcripts regulated by Gcn4p in response to amino acid stress (Figure 4C), consistent with reduced activation of the GAAC response.

PheRS editing dependent growth defects are suppressed by GAAC activation

To further investigate defects in GAAC pathway activation in the PheRS QC deficient strain, the conditionally essential pathway components *GCN2* and *GCN4* were removed from the genome and systematically complemented with WT or constitutively active allelic variants. Growth in response to amino acid limitation was monitored using serial dilution spot assays. Under rich growth conditions, where the GAAC is not normally activated, WT and QC deficient cells with and without *GCN2* or *GNC4* displayed similar patterns of growth (Figure 5A–C, top two rows of each panel). During growth on minimal media without amino acid supplementation, only the *gcn4*$^{-}$ strain showed a slow growth phenotype (Figure 5C, top two rows).

As expected, growth of the QC deficient strain in the presence of high levels of Tyr was diminished as compared to the WT PheRS strain. Complementation of WT and QC deficient *gcn2*$^{-}$ strains with plasmids harboring WT alleles of *GCN2* (p722, Figure 5B) restored growth under minimal media growth conditions, while complementation with a constitutively active allele of *GCN2* (*GCN2*^{c}; p914, Figure 5B) restored growth in the QC deficient strain to WT levels under the same growth conditions. Similar growth restoration was observed when chemical activators of the GAAC pathway, 3-AT and Halofuginone, were present in the medium. When constitutively active alleles of *GCN2* were present in the strain, growth overall was expectedly diminished in all conditions due to constitutive activation of the stress response, which reduces the translational capacity of the cell.

As with the *gcn2*$^{-}$ strains, complementation of WT and QC deficient *gcn4*$^{-}$ strains with plasmids containing a WT allele of *GCN4* (p164, Figure 5C) restored growth to levels observed in WT strains grown in the same media. Complementation of WT and QC deficient *gcn4*$^{-}$ strains with a plasmid harboring a constitutively expressed allele of *GCN4* (*GCN4*^{c}; p238, Figure 5C), constructed by removing the inhibitory uORFs in the 5′ UTR, was able to restore growth to WT levels under all growth conditions. The observation that constitutive expression of Gcn4p is able to rescue growth of the QC deficient strain in the presence of high Tyr suggests

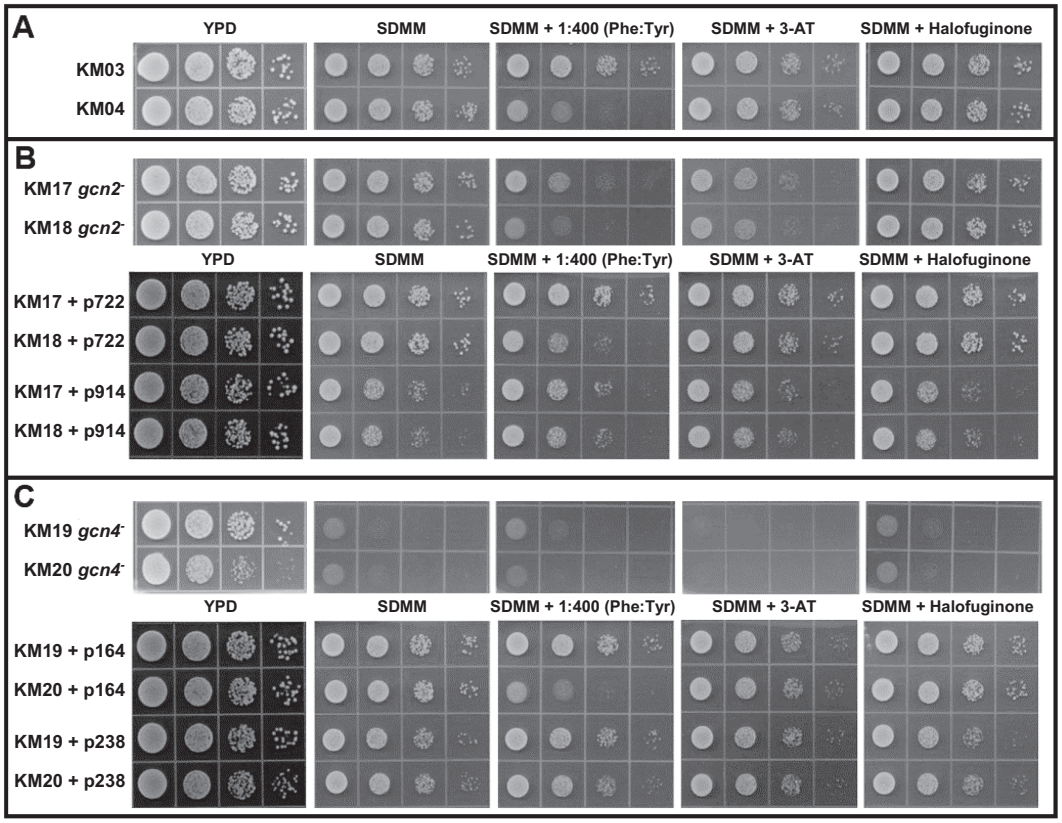

Figure 5. Stress dependent growth of GAAC deficient cells. KM03 (*FRS1*) and KM04 (*frs1-1*) cells with and without chromosomal deletion of GCN2 (KM17 and KM18) or GNC4 (KM19 and KM20) were grown under a variety of growth and stress conditions. 3-AT was supplemented at 10 mM, HF at 10 nM, and Tyr at 1:400 (Phe:Tyr, 1 = 0.003 mM) Deletion strains were transformed with plasmids harboring WT or constitutively active alleles of GCN2 (p722 and p914, respectively) and GCN4 (p164 and p238, respectively) and grown in the same media and stress conditions. Growth was examined after 48 h at 30°C.

that the observed growth defect is at least partly due to deficiencies in the activation of the GAAC pathway.

Tyr-tRNA^{Phe} accumulation leads to increased mistranslation under stress conditions

In addition to its impact on GAAC activation, the accumulation of Tyr-tRNA^{Phe} in the QC deficient strain could potentially lead to significant mistranslation of Phe codons as Tyr. Lack of aaRS editing activity has been proposed to lead to mistranslation of the genetic code, although exact quantification of misincorporation events has rarely been determined (33,34). To quantify the degree to which misaminoacylated Tyr-tRNA^{Phe} accumulation (Figure 1) correlates with mistranslation of Phe codons, we designed a mass spectrometry-based yeast reporter specific for translation of the Phe UUU codon. The reporter was constructed based on an elastin-like polypeptide sequence containing a VPGXG repeat where X has been shown to be highly permissive to any amino acid. We previously utilized these peptide sequences and a similar LC–MS/MS based approach to quantify differences in natural and unnatural amino acid incorporation into proteins (35). The MS reporter segment of the ORF contains a single Phe codon, allowing for the measurement of relative amino acid misincorporation frequencies at that position. Phe codon specific Tyr misincorporation was observed in all samples. Reporter peptides isolated from WT and PheRS editing deficient cells grown in minimal medium showed 0.02% and 0.09% Tyr misincorporation, respectively. The WT misincorporation rate is consistent with previously reported basal translational error rates (36). Tyr misincorporation in WT cells grown in medium containing high levels of Tyr increased to 0.3%, while Tyr misincorporation in editing deficient cells increased to 8% (Figure 6). These data demonstrate that during growth under stress conditions, the absence of PheRS editing of misaminoacylated tRNA^{Phe} results in a 27-fold increase in the rate at which Phe codons are mistranslated as Tyr.

Tyr-tRNA^{Phe} accumulation does not lead to activation of protein stress responses

During growth in excess Tyr and limited Phe, the PheRS QC deficient strain showed a significant increase in mistranslation compared to wild type, which could potentially lead to a substantial increase in the synthesis of misfolded and/or unfolded proteins. In yeast, aberrantly synthesized proteins are targeted by a number of stress response pathways, including the unfolded protein response (UPR) and heat shock family chaperones. In the UPR pathway (UPR-L), Ire1p is activated upon ER stress and mediates the splic-

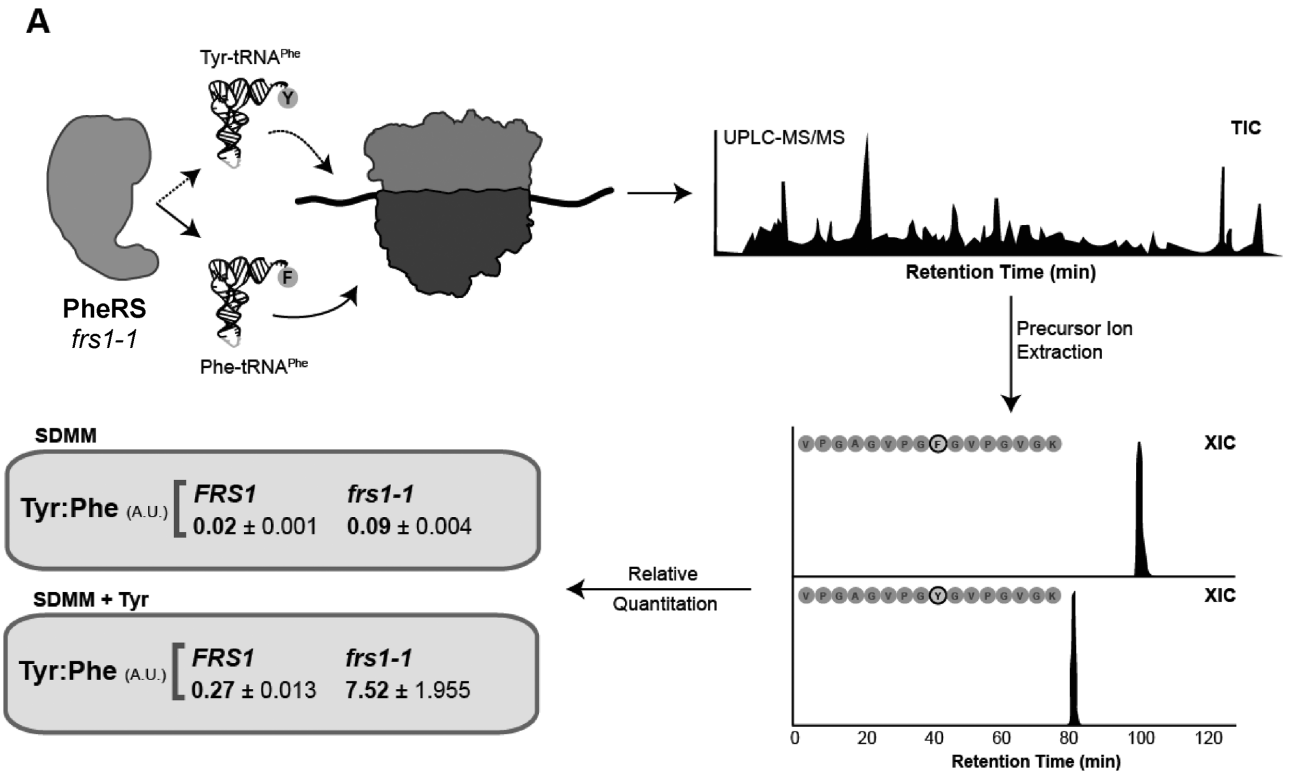

Figure 6. Quantification of Tyr misincorporation at Phe codons. Tyr misincorporation at Phe codons was monitored by mass spectrometry. Reporter protein was expressed recombinantly in KM03 (*FRS1*) and editing deficient KM04 (*frs1-1*) strains grown in minimal medium (SDMM) with and without Tyr supplementation. The reporter peptide was purified from each sample and subjected to mass spectrometry analysis. Data is representative the ratio of Tyr to Phe containing target peptides from two independent experiments.

ing of the transcription factor *HAC1* transcript. Activation of the UPR-L can be assessed by monitoring the splice status of *HAC1* mRNA (37,38). Both the WT and editing deficient strains failed to activate the UPR-L in minimal media and minimal media supplemented with excess Tyr, while Tunicamycin was able to activate the UPR-L in both strains (Figure 7A and B). These results suggest that amino acid dependent growth deficiency observed in the editing deficient strain is not a direct result of global accumulation of mistranslated unfolded proteins in the ER. Additionally, induction of the cytosolic UPR component (UPR-cyto) *SSA4* was assessed by qRT-PCR. *SSA4* encodes the cytosolic chaperone Hsp70p, the main stress inducible chaperone in the cytosol (39,40). Growth of the PheRS editing deficient strain in high Tyr medium failed to activate the UPR-cyto response, as measured by *SSA4* induction (Figure 7C). Transcript levels for the heat shock family stress response protein HSP104, known to respond to defects in protein folding, were characterized by RT-qPCR (40,41). No increase in HSP104 steady state transcript levels was observed for WT or the editing deficient strain in response to growth in minimal media or media supplemented with excess Tyr, (Figure 7D). These data indicate that despite a significant increase in the rate of mistranslation during growth under amino acid limitation, the absence of PheRS QC does not result in a sufficient increase in protein misfolding to activate the corresponding stress responses.

DISCUSSION

Accumulation of misaminoacylated tRNA^{Phe} prevents proper activation of the GAAC pathway

Yeast cells assess the status of intracellular amino acid pools indirectly by monitoring tRNA aminoacylation levels via the GAAC pathway. The GAAC response uses deacylated tRNAs as signaling molecules to indicate stress, which provides a means to both accurately gauge the translational capacity of the cell and detect changes in the growth environment (42). The GAAC response is activated by various stressors and here we demonstrate that one of these, amino acid starvation, can only be accurately sensed by the cell when non-cognate aa-tRNA editing is active. The absence of editing disrupts the cell's ability to sensitively respond to nutritional stress, which depends on deacylated tRNA levels accurately reflecting the availability of the corresponding cognate amino acids (Figure 8). The net result is that PheRS QC deficient cells grow more slowly than wild type during amino acid starvation, presumably due to their inability to adequately increase Phe synthesis via the GAAC pathway, as exemplified by suppression of *ARO3* transcription (Figure 4C). *ARO3* encodes DAHP synthase, which catalyzes the first step in aromatic amino acid biosynthesis in yeast. The insensitivity of the PheRS QC deficient strain to cognate amino acid starvation was circumvented either by supplementation with exogenous Phe or constitutive induction of *GCN2* or *GCN4*, further supporting the role of

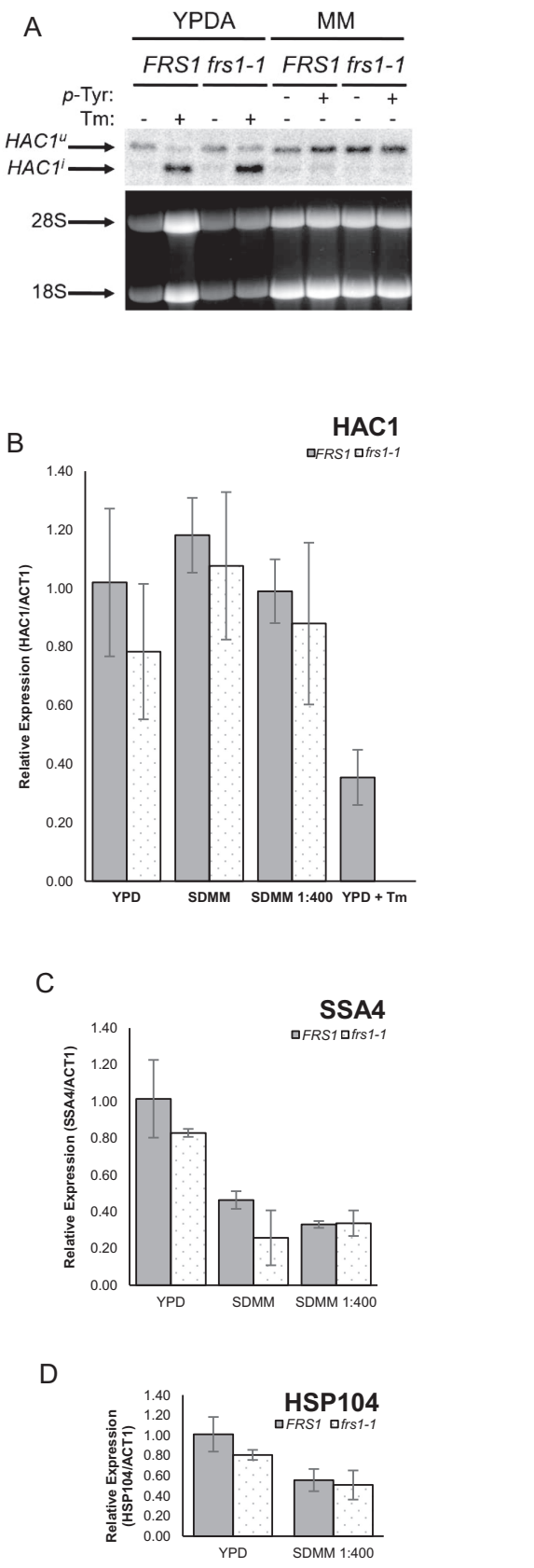

Figure 7. Activation of protein stress response. (**A**) Strains NR1 (*FRS1*) and NR2 (*frs1-1*) were inoculated into 50 ml liquid YPDA, minimal media (MM) + Phe:Tyr 1:1 (0.003 mM Phe, 0.003 mM Tyr), or minimal media (MM) + Phe:Tyr 1:50 (0.003 mM Phe, 0.15 mM Tyr) and grown to an OD600 of 0.8. Cells were harvested by centrifugation, washed with 50 ml dH_2O, frozen, and stored at –80°C overnight for RNA extraction. The unfolded protein response was induced by treating cultures at an OD600 of 0.4 with Tunicamycin (10μg/mL), grown to OD600 of 0.8 and processed as above. Total cellular RNA was extracted using hot phenol. Northern blot analysis was carried out using the NorthernMax –Gly Kit (Ambion) according the manufacturer's instructions. Agarose gels were loaded with 5 μg of total RNA for each sample. A single stranded DNA probe (5′-CAAACAAATTGTTGTTGTCTACGGCAGGTAG-3′) which binds to base pairs 529–559 of the *HAC1* transcript was radiolabeled with [γ-32P]-ATP by T4 polynucleotide kinase and utilized in hybridization. Transcript abundance for protein stress response transcripts HAC1 (**B**), SSA4 (**C**) and HSP104 (**D**) were analyzed by qRT-PCR. cDNA libraries were prepared from 500 ng total RNA extracted from KM03 (*FRS1*) or KM04 (*frs1-1*) cells grown to an OD600 of 0.8–1.0 in minimal medium (SDMM) or SDMM supplemented with 1:400, Phe:Tyr (1 = 0.003 mM). cDNA was analyzed by qPCR using SYBR Green I. Samples were normalized internally to levels of ACT1 and reported relative to WT expression in rich medium (YPD). Error bars represent S.D. from at least three independent experiments.

misaminoacylated-tRNA editing in tuning the sensitivity of the GAAC response (Figure 8). A role for PheRS QC in activation of amino acid starvation responses was first reported for *Escherichia coli* grown in the presence of the cytotoxic non-protein amino acid *meta*-Tyr, where the absence of misacylated tRNA editing delayed transcription of the gene encoding chorismate mutase (10,21). Taken together with previous findings, our data show that PheRS QC plays similar roles in bacteria and in eukaryotes, albeit via regulation of different steps in aromatic amino acid biosynthesis and in response to both non-proteinogenic and proteinogenic non-cognate amino acids, respectively.

Codon specific mistranslation by Tyr-tRNA^{Phe} is not cytotoxic

Errors in protein synthesis arise from a variety of sources including inaccurate transcription, aberrant mRNA processing, premature translation termination, peptide misfolding, and amino acid misincorporation. Amino acid misincorporation is perhaps the most prevalent cause of protein synthesis errors and is a result of either decoding errors at the ribosome or tRNA misaminoacylation. To determine the impact of PheRS QC in preventing tRNA misaminoacylation and how this, in turn, effects protein synthesis accuracy, we quantified tRNA^{Phe} cognate and non-cognate aminoacylation in tandem with amino misincorporation rates. A 5-fold increase compared to wild type in misaminoacylated tRNA accumulation was observed in the PheRS QC deficient strain subjected to high Tyr stress, with Tyr-tRNA^{Phe} accounting for 5.3 ± 0.5% of the cellular aminoacylated tRNA^{Phe} pool. The elevated level of tRNA misaminoacylation observed in the absence of editing correlated well with mistranslation rates under the same conditions, with 7.5 ± 2% of Phe codons being translated as Tyr. Taken together these data show that proofreading of misaminoacylated Tyr-tRNA^{Phe} by PheRS is the primary QC checkpoint that prevents mistranslation of Phe codons during growth under amino acid stress conditions.

Despite mistranslation of Phe codons rising to over 7%

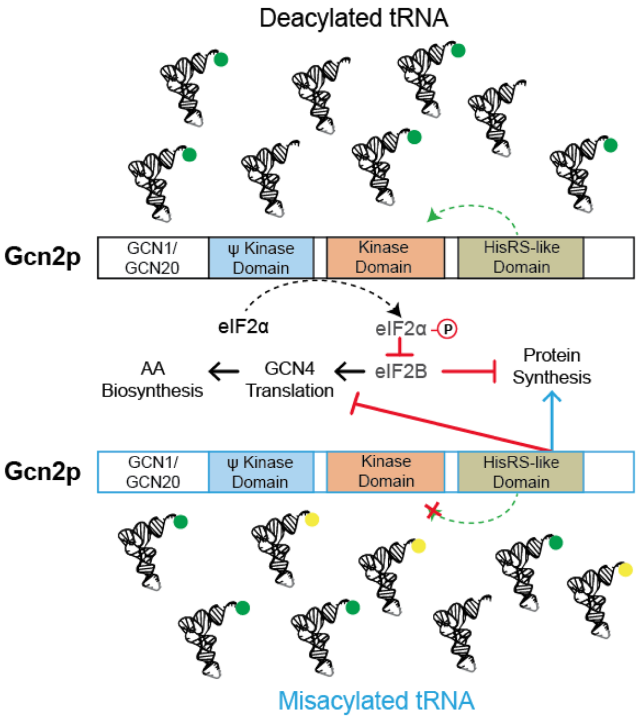

Figure 8. The impact of aa-tRNA on the regulation of cellular stress response. Canonical response to amino acid starvation involves the accurate sensing of deacylated tRNA by Gcn2p. As deacylated tRNA binds, the kinase domain of Gcn2p activates, phosphorylating the guanine exchange factor eIF2α. Phosphorylation of eIF2α results in a reduction in ternary complex levels and subsequent reduction in translation initiation. Lower levels of translation initiation allows for the expression of the transcription factor Gcn4p and cellular reprogramming in response to stress. Misaminocylated tRNA (blue) prevents accurate sensing of starvation by reducing the pool of deacylated tRNA available to interact with Gcn2p. The result is a decrease in GCN4 translation and response to amino acid stress.

in the absence of PheRS QC, this does not lead to activation of unfolded protein stress responses. Previous studies in aa-tRNA editing deficient strains of *E. coli* showed mistranslation rates as high as 10% may cause no adverse effects on growth (43). Even under optimal growth conditions, nonsense suppression of stop codons in wild type *B. subtilis* occurs at a rate of 0.4% (44). Overall, organism-specific experimental measurements of mistranslation range from 0.001 to 10%, with an estimated 15% of all proteins in the cell possessing at least one misincorporated amino acid (reviewed in (45)). It is estimated that 50% of amino acid misincorporation events affect protein stability, often leading to protein misfolding, yet the impact of specific natural amino acid substitutions on overall proteome stability remains largely unknown (46). Despite comparatively high levels of Tyr misincorporation in the absence of PheRS QC, the protein stress response remains inactive indicating that Phe to Tyr mistranslation is well tolerated in yeast. Conversely, in similar growth conditions an *E. coli* strain deficient in Tyr-tRNA^{Phe} editing triggered activation of several protein stress response pathways (10). The divergence in these responses to Tyr misincorporation may reflect divergent active site topologies of the yeast and bacterial PheRSs. Unlike yeast cytoplasmic PheRS, the bacterial homologues of PheRS are able to prevent Tyr misacylation through high level discrimination within the amino acid binding pocket of the active site. All fungal, and some higher eukaryote, cytosolic PheRS enzymes possess a natural Ala to Gly substitution within the active site that enlarges the amino acid binding pocket sufficiently for near cognate amino acids to be efficiently activated (22). The lower amino acid specificity of yeast PheRS inversely correlates with the organism's ability to tolerate protein stress resulting from Tyr misincorporation. The opposite is true for *E. coli,* which has a more discriminating PheRS and is less tolerant of mistranslation (10). Through comparison of these systems, it becomes clear that the level of amino acid misincorporation is less important than the nature of the substitution itself. In yeast, 7% *para*-tyrosine substitution at Phe codons is well tolerated, yet 1% misincorporation of *meta*-tyrosine in *E. coli* results in activation of protein stress responses and severe growth defects (10). These observations show that protein error rates vary substantially from system to system and that, beyond the level of error, amino acid specific chemicophysical properties play critical roles in determining the cytotoxicity of mistranslation.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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