Published online 11 July 2018

Nucleic Acids Research, 2018, Vol. 46, No. 15 ***7831–7843***
doi: 10.1093/nar/gky623

Conditional accumulation of toxic tRNAs to cause amino acid misincorporation

Stephanie M. Zimmerman[1,†], Yoshiko Kon[2,†], Alayna C. Hauke[2,†], Bianca Y. Ruiz[1], Stanley Fields[1,3,4,*] and Eric M. Phizicky[2,*]

[1]Department of Genome Sciences, University of Washington, Seattle, WA 98195, USA, [2]Department of Biochemistry and Biophysics and Center for RNA Biology, University of Rochester School of Medicine, Rochester, NY 14642, USA, [3]Department of Medicine, University of Washington, Seattle, WA 98195, USA and [4]Howard Hughes Medical Institute, University of Washington, Seattle, WA 98195, USA

Received May 18, 2018; Revised June 22, 2018; Editorial Decision June 26, 2018; Accepted July 01, 2018

ABSTRACT

To develop a system for conditional amino acid misincorporation, we engineered tRNAs in the yeast *Saccharomyces cerevisiae* to be substrates of the rapid tRNA decay (RTD) pathway, such that they accumulate when RTD is turned off. We used this system to test the effects on growth of a library of $tRNA^{Ser}$ variants with all possible anticodons, and show that many are lethal when RTD is inhibited and the tRNA accumulates. Using mass spectrometry, we measured serine misincorporation in yeast containing each of six tRNA variants, and for five of them identified hundreds of peptides with serine substitutions at the targeted amino acid sites. Unexpectedly, we found that there is not a simple correlation between toxicity and the level of serine misincorporation; in particular, high levels of serine misincorporation can occur at cysteine residues without obvious growth defects. We also showed that toxic tRNAs can be used as a tool to identify sequence variants that reduce tRNA function. Finally, we generalized this method to another tRNA species, and generated conditionally toxic $tRNA^{Tyr}$ variants in a similar manner. This method should facilitate the study of tRNA biology and provide a tool to probe the effects of amino acid misincorporation on cellular physiology.

INTRODUCTION

For translation to proceed with accuracy, multiple processes must occur with high fidelity. These include quality control steps that ensure accurate translation initiation (1), charging of each tRNA by its cognate aminoacyl tRNA synthetase (aaRS) (2), and decoding of the mRNA codon in the A site of the ribosome by selection of cognate charged tRNAs (3–5). tRNA charging accuracy is ensured by multiple nucleotide determinants and antideterminants in the tRNA sequence, as well as post-transcriptional modifications (6–10) and editing mechanisms (11–14). Failure of quality control at the tRNA charging step is highly likely to lead to misincorporation of an incorrect amino acid. Unlike the quality control steps that ensure accurate decoding by cognate tRNA in the ribosome A-site due to codon–anticodon pairing and related tRNA recognition mechanisms (15–20), no mechanism prevents mischarged tRNA from decoding the A-site codon. Indeed, charging defects lead to a number of human diseases (21–24).

Misincorporation during protein synthesis occurs at a rate of 10^{-3} to 10^{-5} per codon (25–27), ensuring that most typically-sized proteins are accurately made (28). Misincorporation can lead to the accumulation of misfolded or aggregated proteins and proteotoxic stress, and has been linked to disease (29,30). However, in some cases, high rates of misincorporation are tolerated. For example, an *Escherichia coli* mutant that translates 10% of asparagine sites as aspartic acid has no obvious growth defects (31). In *Candida* species, the CUG codon is decoded as serine instead of leucine (32–34), but the *Candida* $tRNA^{Ser(CAG)}$ can be mischarged by LeuRS, resulting in 3–4% leucine misincorporation in wild type cells (35). This misincorporation can be boosted to 28% upon expression of a $tRNA^{Leu(CAG)}$ variant without obvious growth defects (35). Similarly, high levels of misincorporation with limited growth defects have been measured in engineered *Mycobacterium smegmatis* (36), as well as in wild type *Mycoplasma mobile* (37) and *Bacillus subtilis* (38).

Misincorporation has also been harnessed as a tool to expand the genetic code and to impart new functions to proteins. Engineered tRNA and tRNA synthetase pairs have been used to incorporate multiple types of non-

*To whom correspondence should be addressed. Tel: +1 585 275 7268; Email: eric_phizicky@urmc.rochester.edu
Correspondence may also be addressed to Stanley Fields. Email: fields@uw.edu
†The authors wish it to be known that, in their opinion, the first three authors should be regarded as Joint First Authors.

canonical amino acids, including those that are fluorescent, post-translationally modified, or chemically reactive. These tRNA/synthetase pairs have generally been designed to act at a termination codon and to use a nonsense suppressor tRNA (reviewed in (39)). The success of this approach indicates that incorporation of non-canonical amino acids is typically tolerated, at least at termination codons.

Much remains to be learned about the process and effects of amino acid misincorporation. It is not yet clear what levels of misincorporation cause toxicity, how the level of misincorporation depends on the identity of the amino acid and the codon, and how different types of substitutions affect fitness. New tools to engineer misincorporation would enable studies of the biological effects of translational errors, and would facilitate the development of tRNAs for genetic code expansion. However, a major limitation in studies of eukaryotic tRNA biology is a lack of effective methods to regulate tRNA levels in cells, in part because tRNA genes have internal pol III promoters that comprise part of the mature tRNA sequence (40–42). This lack of methods prevents the conditional expression of lethal or toxic tRNA variants.

To address these questions, we established a system in the yeast *Saccharomyces cerevisiae* to conditionally accumulate tRNAs by engineering tRNAs to be substrates of the rapid tRNA decay (RTD) pathway, and then shutting off the RTD pathway. The RTD pathway degrades a number of specific hypomodified tRNA species, including $tRNA^{Val(AAC)}$ lacking 7-methylguanosine and 5-methylcytidine (43); and $tRNA^{Ser(CGA)}$ and $tRNA^{Ser(UGA)}$ lacking either 4-acetylcytidine and 2′-*O*-methyluridine, or 2′-*O*-methylguanosine and 5-methylcytidine (44–46). Other substrates of the RTD pathway include fully modified $tRNA^{Ser(CGA)}$ variants with destabilizing mutations in the acceptor stem and T-stem (47), and fully modified $SUP4_{oc}$ variants (derived from $tRNA^{Tyr}$) with destabilizing mutations in any of the stems or loops (48,49). In all tested examples, RTD is enhanced at 35–37°C relative to 28°C, but decay is observed throughout the temperature range (44–47,49). We use this system to conditionally misincorporate amino acids into proteins using RTD-sensitive $tRNA^{Ser(CGA)}$ variants that can insert serine at non-serine codons. We comprehensively assess the toxicity of this serine tRNA with all possible anticodon sequences, and demonstrate that toxicity and misincorporation levels vary widely depending on anticodon. Surprisingly, cells are almost completely tolerant of high levels of misincorporation of serine at cysteine codons. Finally, we demonstrate the utility of conditional accumulation of toxic tRNAs to investigate sequence determinants of tRNA function.

MATERIALS AND METHODS

Construction of yeast strains

Strain YK380-1 (BY4741 *can1::MET15::GFPoc met22Δ::HygR*) was described previously (48), and was transformed with a *[URA3 CEN MET22]* plasmid to generate YK1311-1. The *MET22* gene of strain BY4741 (*MAT***a** *his3-Δ1 leu2-Δ0 met15-Δ0 ura3-Δ0*) and JMW223 (BY4741 $tS(CGA)\Delta::ble^R$ [*CEN URA3 tS(CGA)*]) (47) was placed under control of the P_{GAL1} promoter using the cassette plasmid pYM-N22 (KanMX4::P_{GAL1}) (50) to generate strains YK360-1 and YK362-1.

To test the suitability of the tS(CGA)-C3U-U6C (tS*(CGA)) variant as a scaffold for RTD, we integrated this tS*(CGA) variant or the control WT tS(CGA) into YK362-1 with an adjacent *MET15* marker at the *ADE2* locus (47). Purified transformants were grown overnight on YP medium (1% yeast extract, 2% peptone, supplemented with 20 mg/l adenine hemisulfate) containing 2% glucose (D), and plated on synthetic (S) medium containing 5-fluoroorotic acid at 25°C to select cells that lost the *URA3* plasmid. The resulting purified Ura^- cells (YK374-1: relevant genotype P_{GAL} *MET22* $tS(CGA)\Delta::ble^R$ $ade2^-$*::tS*(CGA)::MET15*), and control strain YK375-1 were then tested for growth and tRNA levels when *MET22* expression was regulated at different temperatures. The control strain JMW790 (relevant genotype: *met22Δ tS(CGA)Δ tS*(CGA)::MET15*) was described previously (47).

Northern analysis of tRNA levels

To analyze tS*(CGA) and WT tS(CGA) levels, P_{GAL}*MET22* strains or control *met22Δ* strains were grown to log phase at 27°C in YP medium containing raffinose. Galactose or glucose was added to 2%, growth was continued for 8–10 hours, and cells were harvested at an OD_{660} of 1.0–2.0. Bulk RNA was prepared using hot phenol, 1 μg RNA was resolved on a 10% polyacrylamide 7 M urea gel, and RNA was analyzed by Northern blot as previously described (43) using appropriate hybridization probes.

Plasmids

High copy 2μ plasmids were constructed to express a tRNA gene that was cloned from its normal chromosomal locus with flanking regions, or a gene that was constructed with oligomers and inserted into the Bgl II Xho I site of JW132 to express the tRNA gene with the flanking regions of tH(GUG)G2, which was subsequently transferred as needed to 2μ *leu2-d* plasmid (47).

Analysis of toxicity of tS* and tY* variants in a P_{GAL}-*MET22* strain or a *met22Δ* [*URA3 CEN MET22*] strain

To test toxicity of tS* variants on plates, either of two methods was employed. For the first method, the corresponding tS* variant was integrated into YK360-1 with an adjacent *MET15* marker at the *ADE2* locus (47), and transformants were selected on S-Met+Raff+Gal medium at 35°C. Transformants were analyzed for for toxicity on YPD or YP+Raff+Gal media at different temperatures. For the second method, the corresponding tS* variant was integrated into strain YK1311-1 with an adjacent *HIS3* marker at the *ADE2* locus, and transformants were selected on S-His medium at 35°C. Transformants were analyzed for toxicity by overnight growth and plating on media containing 5-FOA or YPD at different temperatures.

To test toxicity of tY*-C(GCA) variants on plates, we integrated strain YK1311-1 with tY*-C(GCA) at *ADE2* with

a *HIS3* marker and/or at *PHA2* with a *KanR* marker. The resulting strains with 0, 1 or 2 integrated copies of tY*-C(GCA) were then grown overnight in YPD media at 35°C, and tested for toxicity by serial dilution and plating on media containing 5-FOA at different temperatures.

Variants were scored on a 0–5 scale (with 0 being most toxic) based on an arbitrary growth assessment relative to non-toxic controls. The results agreed when independently assessed by two parties.

Analysis of tRNA suppressors of the toxicity caused by tS*-F(GAA) and tS*-I(GAU)

To isolate suppressors of the toxicity conferred by tS*-F(GAA), we constructed strain YK1326-3 by integrating tS*-F(GAA) into YK1311-1 at the *LYS2* locus with an adjacent *LEU2* marker (generating *lys2::tS*-F(GAA)::LEU2*), followed by integration of $SUP4_{oc}$-*C5U* at the *ADE2* locus with a *HIS3* marker (*ade2::*$SUP4_{oc}$*::HIS3*) to generate YK1356-1. Strain YK1368-1 was generated in an analogous manner, containing *lys2::tS*-I(GAU)::LEU2* instead of the corresponding tS*-F(GAA). Spontaneous suppressors of the toxicity were then selected by growing cells overnight in YPD at 35°C and plating on media containing 5-FOA at 30°C and 25°C, and the toxic tRNA at the *LYS2* locus was PCR amplified and sequenced.

Construction and selection of the tS*(CGA)-NNN anticodon library

A pool of plasmids containing tS*(CGA) with all possible anticodon sequences was constructed by site-directed mutagenesis using a degenerate oligo with NNN at the anticodon, using plasmid AB254 as template. A fragment containing the tRNA, *MET15* selectable marker, and flanking *ADE2* homology was amplified by PCR and transformed into the P_{GAL}-*MET22* strain using a high-efficiency protocol (51), resulting in ∼3000 transformants. The transformation was plated on S-Met+Gal plates and grown at 35°C for 4 days. Cells were collected from the plates and a sample was used to seed 100 ml liquid cultures grown with shaking in YPGal media at 35°C or YPD media at 30°C for 16 h. The remaining cells from the plates and the liquid cultures were harvested for genomic DNA isolation and sequencing.

Sequencing and sequencing data analysis

The tS*(CGA) gene was amplified from miniprepped plasmid or yeast genomic DNA using Phusion high-fidelity polymerase (New England Biolabs) and primers that added Illumina adaptor sequences and sample index sequences. Amplification was monitored by qPCR and all libraries were amplified for fewer than 15 cycles to avoid artifacts due to overamplification. PCR products were cleaned using a DNA Clean and Concentrater-5 kit (Zymo Research). The amplicons were sequenced on an Illumina Nextseq to generate 75 bp paired end reads spanning the entire sequence of the tRNA. Reads were mapped to the tS*(CGA) sequence using Bowtie2 (52). Reads with gaps or mismatches to the tRNA gene outside of the anticodon were removed, as were reads in which the anticodon sequence did not agree between read 1 and read 2. After these filtering steps, ∼500 000 reads remained for each condition. The frequency of each anticodon in the yeast samples was normalized to its frequency in the plasmid pool before transformation into yeast.

For the analysis correlating codon usage with toxicity, we counted instances of each codon in all open reading frames (ORFs) annotated in the *Saccharomyces* Genome Database (SGD) and divided by the total number of codons in yeast ORFs. To estimate usage of each codon in the proteome, we multiplied each instance of a codon by the steady state expression level of its corresponding protein, as estimated by Ghaemmaghami *et al.* (53). The set of codons that can be decoded by each anticodon was defined by Johansson et al (54).

Sample preparation for mass spectrometry

Overnight cultures of tRNA variants grown in YPGal media at 35°C were used to start 10 ml cultures in YPD media grown at 30°C. Cultures were diluted every 24 h to fresh YPD media and harvested after 72 h. A sample of each culture was used for growth assays, in which growth was monitored every 15 min by absorbance at OD_{660} on a BioTek Synergy H1 plate reader.

Cell pellets from the resulting 10 mL of yeast culture were resuspended in a denaturing lysis buffer (8 M urea, 50 mM Tris, pH 8.2 and 100 mM NaCl). Cells were lysed by bead beating with 0.5 mm glass beads at 4°C. Lysates were cleared by centrifugation at 10 000 × g for 10 min at 4°C. The Pierce BCA Protein Assay kit (Thermo Fisher Scientific) was used to determine protein concentration. Proteins were reduced with 5 mM dithiothreitol (DTT) for 45 min at 55°C, alkylated with 15 mM iodoacetamide for 45 min at room temperature, and quenched with 5 mM DTT for 15 min at room temperature. For each sample, 100 μg of protein was diluted 1:2 with 50 mM Tris, pH 8.9 and digested overnight at room temperature with 10 ng/μl LysC (Wako Chemicals). Digests were acidified to pH 2 with TFA and desalted using stage tips constructed from Empore C18 wafers (3M) (55). Peptides were dried and resuspended in 5% acetonitrile, 5% TFA for mass spectrometry.

Mass spectrometry

Peptides were separated by a liquid chromatography gradient on an EASY-nLC-1000 (Thermo Fisher Scientific). Eluted peptides were analyzed online with a hybrid quadrupole-orbitrap Q-Exactive mass spectrometer (Thermo Fisher Scientific) using data-dependent acquisition in which the 20 most abundant ions on an MS scan were selected for fragmentation by beam-type high-energy collisional dissociation (HCD), and fragmented ions were excluded from further selection for 40 s. Full MS scans were acquired in centroid mode from 300 to 1500 m/z at 70 000 full width at half-maximum (fwhm) resolution with a maximum injection time of 100 ms and fill target of 3×10^6 ions. MS/MS fragmentation spectra were collected at 17 500 fwhm with maximum injection time of 50 ms, using a 2.0 m/z precursor isolation window and fill target of 5×10^4 ions. Acquisition time for each sample was 120 min.

MS data analysis

Raw spectra were converted to the mzXML format and searched against the SGD yeast protein sequence database (6717 entries) using Comet (56). The false discovery rate was estimated using target-decoy searching by searching a concatenated forward and reverse version of the database (57), and filtered using Percolator (58) to a 1% false discovery rate (FDR) at the peptide-spectrum match (PSM) level. The maximum FDR was less than 1.5% at the peptide level. To identify peptides with serine substitutions at a given amino acid, the search was conducted with a variable modification corresponding to the mass shift of a serine substitution at each instance of the target amino acid. A maximum of two serine substitutions per peptide were allowed, as the low rate of substitution we observed was considered unlikely to produce detectable peptides with multiple substitutions. Additional search parameters were: digestion at lysine with a maximum of two missed cleavages, a constant modification of cysteine carbamidomethylation, variable modifications of N-terminal acetylation and methionine oxidation, precursor mass tolerance of 50 ppm, fragment bin tolerance of 0.02 Da, and fragment bin offset to 0, as recommended for high resolution MS1 and MS2 instruments (59).

To measure the frequency of substitution, we calculated the fraction of unique peptides containing the target amino acid for which the serine-substituted version of the peptide was also detected. Serine-substituted peptides with additional modifications (methionine oxidation or N-terminal acetylation) or those in which the corresponding wild-type peptide was not present in the dataset were filtered out, as they were deemed more likely to be misidentifications. For the analysis of which codons had serine substitutions, only peptides with a single instance of the relevant amino acid were included to eliminate the possibility of incorrect site localization of the substitution in the peptide.

RESULTS

To construct a system in yeast for regulated accumulation of mistranslating tRNAs, we focused on manipulation of $tRNA^{Ser}$. The seryl-tRNA synthetase (SerRS) uses the unique variable arm of $tRNA^{Ser}$ as a major identity element for charging (60), and therefore its serine charging efficiency is unaffected when the $tRNA^{Ser}$ anticodon is changed. To design a system that allows the conditional accumulation of otherwise lethal $tRNA^{Ser}$ variants, we constructed $tRNA^{Ser(CGA)}$ (called tS(CGA)) variants with non-serine anticodons under the control of the RTD pathway (Figure 1A). Since tS(CGA) was known to be degraded by the RTD pathway if it had destabilizing mutations in the acceptor and/or T stem (47), we tested the suitability of a tS(CGA) variant with acceptor stem mutations C3U and U6C (called tS*), which we previously identified as an RTD substrate in the temperature range 25°C–33°C (47). We integrated either tS*(CGA) or wild type tS(CGA) into yeast in which the only chromosomal copy of the essential gene encoding tS(CGA) was deleted and in which RTD could be regulated. Since a *met22Δ* mutation inhibits RTD in all known cases (45–49), we placed the chromosomal *MET22* gene in this strain under galactose control by insertion of a promoter construct (50). The resulting P_{GAL} *MET22 tS(CGA)Δ tS*(CGA)* strain grew on glucose-containing media at 25, 30 or 33°C, but grew poorly or died at 30 and 33°C on galactose-containing media, in which *MET22* was expressed (Figure 1B). Consistent with this growth phenotype, tS*(CGA) levels were reduced in galactose relative to glucose at 27°C, and were further reduced at 33 and 33.5°C (Supplementary Figure S1A). In contrast, the levels of WT tS(CGA) appeared unaffected in the P_{GAL} *MET22* strain in either media at any temperature tested, and the levels of tS*(CGA) were similar in either media in a *met22Δ tS(CGA)Δ tS*(CGA)* strain. These results indicate that tS*(CGA) is conditionally degraded by restoration of the RTD pathway in galactose, particularly at higher temperatures, allowing control over tRNA levels.

Next, using the P_{GAL}-*MET22* strain, we integrated tS* variants with either a GAA anticodon or GAU anticodon, which are predicted to insert serine at phenylalanine codons (UUU or UUC) or isoleucine codons (AUC or AUU), respectively (Figure 1C). Yeast expressing tS*-F(GAA) or tS*-I(GAU) grew on galactose-containing media in which Met22 is expressed and the RTD pathway is active, but died on glucose-containing media in which Met22 is not expressed, RTD is inhibited, and the tRNAs accumulate (Figure 1C). The lethality of tS*-F(GAA) could be suppressed by overproduction of tF(GAA), but not tS(CGA), tW(CCA), tI(UAU), or tI(AAU), suggesting that toxicity is due to insertion of serine at phenylalanine codons. Similarly, the lethality of tS*-I(GAU) could be suppressed by tI(AAU) but not tI(UAU) or three other control tRNAs, consistent with toxicity due to serine insertion at AUC or AUU codons (Figure 1C). Both tS*-F(GAA) and tS*-I(GAU) were considerably more toxic at 25°C than 30°C or 35°C, consistent with the increased stability of RTD substrates at lower temperatures, but toxicity was still almost fully suppressed by overproduction of a competing tRNA (Supplementary Figure S1B).

Conditional accumulation of a toxic tRNA could also be achieved in a *met22Δ* [*CEN URA3 MET22*] strain, in which the only copy of *MET22* is carried on a *URA3* low copy plasmid. Yeast expressing tS*-F(GAA) or tS*-I(GAU) in this background grew well on YPD media at 30–37°C, but grew slowly or not at all at 25 and 30°C on media containing 5-fluoroorotic acid (5-FOA), which selects for loss of the *URA3* plasmid (Figure 1D). From this assay, we observed that the tS*-I(GAU) variant was more toxic than the toxic tS*-F(GAA) variant, and was toxic even without selection against *MET22* at 25°C.

To explore the extent to which different anticodon variants of tS* cause toxicity, we generated a library of tS* genes with all 64 possible anticodons in the context of adenosine at residue 37 immediately 3′ of the anticodon. Residue 37 of tRNAs is almost universally a purine, and in yeast 31 of the 41 natural tRNA anticodons are followed by A37. Mutations in the anticodon are not known to affect RTD, since they do not affect tRNA stability. We integrated this library into yeast at the *ADE2* locus, and sequenced the population of tS* variants at three stages: immediately after transformation on S-Met+Gal media at 35°C; after outgrowth on YPGal media at 35°C; and after outgrowth on YPD media at 30°C (Figure 2A). Cells expressing toxic tS* variants should be depleted after outgrowth in YPD media at 30°C,

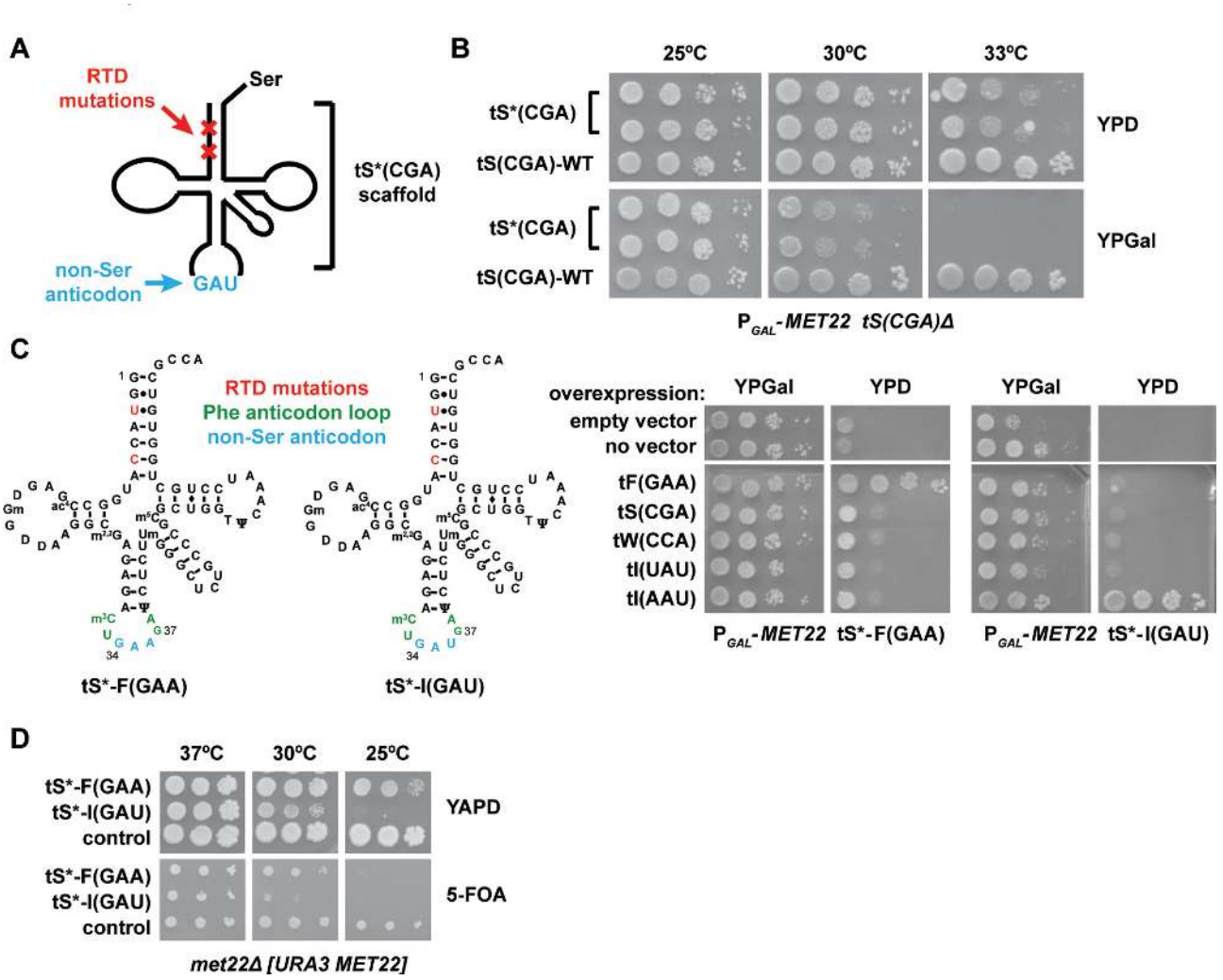

Figure 1. Design and demonstration of a conditionally toxic tRNA. (**A**) Schematic of the design of a conditionally toxic tRNA. Mutations in the acceptor stem (red X's) make $tRNA^{Ser(CGA)}$ a substrate of the rapid tRNA decay (RTD) pathway. Altering the anticodon to that of a non-serine codon (eg., GAU, blue) causes serine misincorporation and toxicity. (**B**) Demonstration that the tS* scaffold makes tS*(CGA) a substrate for RTD in a strain with *MET22* under control of the P_{GAL} promoter. An integrated copy of tS*(CGA) fails to complement loss of the wild type copy of tS(CGA) when *MET22* is expressed in the presence of galactose, turning on the RTD pathway. *tS(CGA)Δ::ble*^{R} strains with an integrated *tS*(CGA)* or WT *tS(CGA)* gene as indicated were grown overnight in YPD medium or YP medium containing 2% raffinose and 2% galactose (YPGal), and then serially diluted by factors of 10, and spotted on the same medium at different temperatures to monitor growth. (**C**) Demonstration that tS*-F(GAA) and tS*-I(GAU) are conditionally toxic in a P_{GAL}-*MET22* strain due to misincorporation at Phe and Ile codons respectively. Left: Structure of the conditionally toxic tRNAs tS*-F(GAA) and tS*-I(GAU). Right: Spotting assays showing conditional toxicity of tS*-F(GAA) and tS*-I(GAU) at 30°C and rescue by multicopy expression of tF(GAA) and tI(GAU) respectively. On YPGal medium, *MET22* is induced, the RTD pathway is turned on, and cells expressing tS*-F(GAA) and tS*-I(GAU) survive. On YPD medium, *MET22* is not expressed, the RTD pathway is inhibited, and the tS* variants accumulate and cause toxicity. Overexpression of a wild type tRNA that recognizes the same codons (tF(GAA) and tI(AAU), respectively) rescues the toxicity. (**D**) Demonstration that tS*-F(GAA) and tS*-I(GAU) are conditionally toxic in a *met22Δ* [CEN *URA3 MET22*] strain on media containing 5-FOA. tS*-F(GAA) and tS*-I(GAU) can conditionally accumulate and cause toxicity after loss of a [*URA3 MET22*] plasmid from a *met22Δ* strain.

in which Met22 is not expressed and the toxic tRNA accumulates. Particularly toxic variants that are able to cause toxicity even when Met22 is present might also be depleted immediately after the transformation, or after outgrowth in YPGal at 35°C. To assess the toxicity of the variants and to measure selection that occurred in the presence or absence of Met22, we calculated the change in genotype frequency for each tRNA variant in each growth condition relative to its frequency in the initial plasmid pool before transformation into yeast (Figure 2B, Supplementary Table S1).

Cells expressing tS* anticodon variants of tS* were grown together in a pool, such that those expressing non-toxic tRNA variants would be expected to increase in frequency as they outcompeted cells expressing toxic variants. The results from this competitive selection showed a wide range of toxicity among the anticodon variants. As expected, all anticodons that decode serine codons increased in frequency during the selection, indicating that this $tRNA^{Ser}$ species with a serine anticodon does not cause toxicity. Overall, 35 variants increased in frequency during the selection, indicating that they cause little or no toxicity. Eleven an-

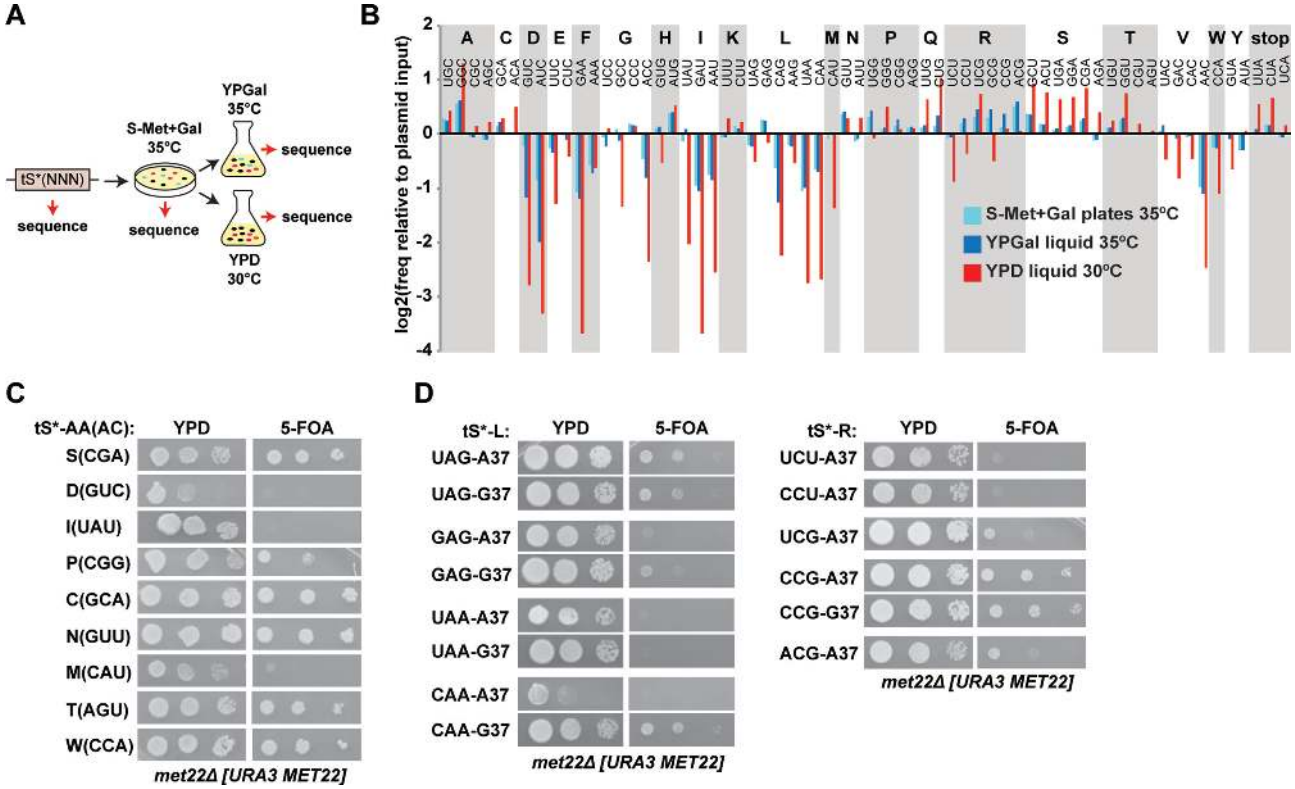

Figure 2. Identification of anticodons that cause toxicity on the tS*(CGA) scaffold. (**A**) Schematic of a competitive selection to define toxic anticodons. DNA containing the *tS*(CGA)* gene with all 64 possible anticodons in the context of A at position 37 was transformed into yeast and selected in either YPGal at 35°C (tRNA degraded) or YPD at 30°C (tRNA accumulates). The pool of variants was sequenced before transformation, after transformation, and after outgrowth in YPD or YPGal. (**B**) Log_2 transformed ratios of the frequency of each anticodon relative to its initial frequency in the DNA before transformation into yeast. (**C**) Assay of individual tS* variants for toxicity at 30°C. Strains of relevant genotype *met22Δ* [*URA3 MET22*] with different integrated tS* variants were grown in YPD medium overnight at 35°C, and serially diluted and spotted at 25°C on YPD or on media containing 5-FOA as indicated. Some tRNAs cause toxicity on 5-FOA media due to loss of the [*URA3 MET22*] plasmid, while others do not. (**D**) Comparison of toxicity of tS* variants with A37 and G37. Strains as described in (C) with integrated tS* variants bearing A37 or G37 as indicated, were grown, serially diluted by factors of 10, and spotted at 25°C on YPD or on media containing 5-FOA to assess toxicity. Left, individual tS*-L variants; right, tS*-R variants.

ticodons were strongly depleted after selection, including tS*-I(GAU) and tS*-F(GAA), while the remaining 18 anticodons had intermediate levels of depletion. Anticodons that became highly depleted during outgrowth in YPD at 30°C were generally also slightly depleted in YPGal at 35°C, suggesting that selection against these variants occurs even in the presence of Met22, but toxicity is much stronger in conditions in which tRNA decay is inhibited.

To directly assess toxicity, we also integrated 41 individual tS* variants with differences in the anticodon at residues 34–36 and in the purine at residue 37, and tested them for growth in the *met22Δ* [*URA3 CEN MET22*] strain after selection on 5-FOA for loss of the plasmid carrying *MET22* (Figure 2C-D, Supplementary Figure S2). Of these variants, 36 had an anticodon that naturally occurs in yeast. Ten variants were made with both A37 and G37 (Supplementary Table S2). Based on the degree of toxicity on 5-FOA media and YPD media at different temperatures, we derived an approximate toxicity score, ranging from 0 (most toxic) to 5 (similar to WT) for each tRNA (see Materials and Methods). For the sake of comparison, we also assigned similar qualitative scores to each anticodon in the competitive selection based on its degree of depletion relative to the starting plasmid pool (Supplementary Table S2). Scores from these individual toxicity assays matched well with the competitive selection experiment at 30°C; 26 of the 31 anticodons tested in both experiments gave similar qualitative scores (Supplementary Table S2). As in the competitive selection, in the absence of Met22 some tS* variants did not detectably affect growth, while others were lethal (Figure 2C). Furthermore, nine variants were substantially more toxic at 25°C in FOA than at 30°C, emphasizing the temperature sensitivity of the tS* scaffold and the ability to tune toxicity using temperature (Supplementary Figure S2).

For some amino acids encoded by multiple codons, certain anticodons led to severe toxicity while others led to much weaker or no toxicity. For example, tS*-L(UAA) and tS*-R(CCU) were highly toxic in the absence of Met22 at 25°C, while tS*-L(UAG) and tS*-R(CCG) were not nearly as toxic (Figure 2D). In some cases, these results can be rationalized by codon usage; for example, CGG (decoded by tS*-R(CCG)) is the rarest arginine codon in the genome and least preferred in highly expressed genes (61). However, tS*-L(UAG) is predicted to decode more prevalent codons and codons that are more preferred in highly expressed genes than tS*-L(GAG), but was much less toxic.

To assess whether serine substitutions at more common or more preferred codons led to greater toxicity, we cor-

related toxicity with codon usage in open reading frames (ORFs) and in the proteome (Supplementary Figure S3A). To estimate the frequency with which each codon is used in the proteome, we multiplied each instance of a codon by the steady state expression level of its corresponding protein, as estimated by the use of fluorescent reporters (53). For both ORFs and the proteome, toxicity correlated poorly with codon usage (Supplementary Figure S3A), suggesting that it is caused by a more complex combination of factors than simply codon frequency. We also investigated whether the identity of the native amino acid compared to serine was a major determinant of toxicity, by correlating toxicity with the substitution probability of each amino acid to serine in homologous sequences (BLOSUM62 score, (62)) (Supplementary Figure S3B). There was a clear trend of substitutions with lower scores (less frequently substituted to serine during evolution) causing greater toxicity, although different substitutions with the same BLOSUM score produced a range of toxicities (Supplementary Figure S3B). These results suggest that the nature of the amino acid change may be a more important driver of toxicity than simply the number of mistranslation events in the proteome.

We observed that regardless of the natural sequence, tRNAs with A37 tended to be more toxic than those with G37. Residue 37 of tRNAs, almost universally a purine, is frequently modified and stabilizes codon:anticodon interactions, and the identity of the purine and of its corresponding modifications varies depending on the sequence of the anticodon (63). Of the ten anticodons that were tested with either A37 or G37, three were substantially more toxic with A37 than G37 at 25°C, one was substantially more toxic with A37 at 30°C, and the other six had similar toxicity scores (Supplementary Figure S2, Supplementary Table S2). This observed toxicity difference did not correlate with the specified amino acid. Natural leucine tRNAs in yeast have a G37 residue (64), but tS*-L(GAG) and tS*-L(CAA) were more toxic with A37 than G37, whereas tS*-L(UAG) was not more toxic with A37, and tS*-L(UAA) was only slightly more toxic with A37 (Figure 2D, Supplementary Figure S2). Similarly, tS*-P(AGG) was substantially more toxic with A37 than G37, whereas tS*-P(UGG) was only modestly more toxic with A37.

The presence of toxic tRNAs exerts strong selective pressure on the cells that express them, and therefore these tRNAs can be used in suppressor screens to identify modifiers of tRNA function. To test this approach, we plated cells expressing tS*-F(GAA) or tS*-I(GAU) on 5-FOA, and isolated spontaneous suppressor colonies that grew on this medium. We sequenced the tRNA variant of the suppressors from both strains and identified 23 mutations in the tS*(CGA) gene (Supplementary Figure S4, Supplementary Table S3). Of these, nine were located in the variable loop of the tRNA, which is necessary for recognition of the tRNA by SerRS (60); 11 others disrupted base pairing in one of the stems, which would lead to instability and reduced function; the U33A mutation alters the nearly invariable U33 anticodon loop residue to the much less functional A33 (65); the A14U mutation disrupts the universally conserved U8-A14 tertiary interaction in tRNA (66); and the G9U mutation is likely to disrupt a base triple with the G13–A22 pair in class II tRNAs, which feature a long variable arm (67). These results demonstrate the utility of using toxic tRNAs to identify functional elements of the tRNA itself, as well as potentially in screens for other genes that act in tRNA biogenesis or function.

To test directly the extent to which toxic tRNAs cause misincorporation, we used mass spectrometry to search for evidence of serine misincorporation in the proteome. We chose six tRNAs to test: four that were toxic to varying degrees (tS*-I(GAU), tS*-D(GUC), tS*-F(GAA) and tS*-P(CGG)) and two that caused little to no toxicity at 30°C (tS*-T(AGU) and tS*-C(GCA)), all in the context of A at position 37. We grew cells expressing these tRNAs in YPD at 30°C for 72 h to ensure that toxic tRNA accumulation and misincorporation had reached steady state, extracted total protein, and analyzed it by liquid chromatography with tandem mass spectrometry (LC–MS/MS). In parallel, we monitored the growth rates of the same cultures during the course of the experiment. To identify putative substitutions, we searched the resulting spectra against the yeast proteome, allowing all sites of the target amino acid to have the wild type mass or a mass shift corresponding to a serine substitution. For each target amino acid, we also searched spectra using the same parameters from control samples expressing tS*(CGA), which has the wild type anticodon. From these results, we could estimate the background rate of detecting a peptide with a serine substitution, likely due to erroneous identifications but also possibly to natural misincorporation events or genetic differences between our strains and the reference genome.

For all of the strains expressing tRNAs with a non-serine anticodon except the one expressing tS*-T(AGU), we identified hundreds of peptides with serine substitutions at the sites of the target amino acid (Supplementary Figure S5A, Supplementary Table S4). In the five strains with detectable misincorporation, we identified serine substitution in ∼2–10% of the peptides with a site for the target amino acid, percentages that were 10 to 100 times higher than in the control samples (Figure 3A). The one exception, tS*-T(AGU), did not have a significantly higher occurrence of Thr to Ser substitution than the control strain. In true examples of misincorporation, we would expect to detect the wild type version of the peptide, as the wild type tRNAs decoding that codon are still expressed and only a fraction of protein molecules would have a serine substitution at any given site. For the five strains with substantial misincorporation, we detected the wild type form of the peptide for a large fraction of the serine-substituted peptides: over 80% for the tS*-F(GAA), tS*-D(GUC), tS*-I(GAU), and tS*-C(GCA) samples, and over 60% for the tS*-P(CGG) samples, which had a higher level of background than the other searches (Supplementary Figure S5A, Supplementary Table S4). The serine-substituted peptides that did not have a wild type form identified were filtered out of the analysis, as they were more likely to be misidentifications.

As an additional control to confirm that the serine substitutions we detected were specific to the tRNA variant expressed, we searched each MS dataset using the same parameters as used for each of the other substitutions. For example, the tS*-F(GAA) MS data were searched in turn for Phe to Ser substitutions, Asp to Ser substitutions, Pro to Ser substitutions, etc. None of the alternative searches

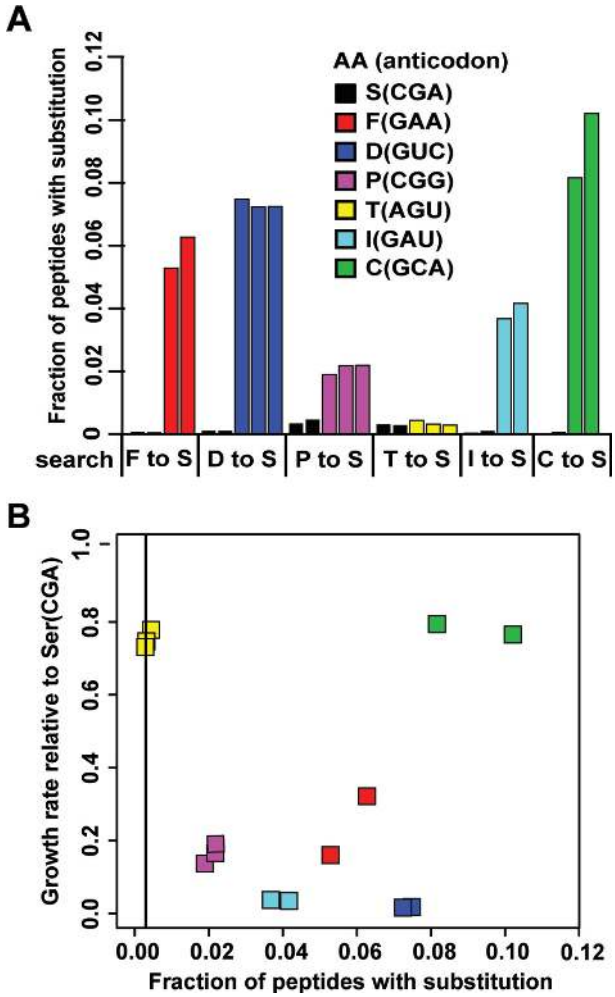

Figure 3. tS* anticodon variants cause serine misincorporation. (**A**) Peptides containing serine substitutions were identified by LC–MS/MS from yeast expressing tS* with the indicated anticodon in the context of A at position 37 and a control strain expressing tS* with the wild type CGA anticodon (black). The fraction of all peptides containing the target amino acid for which a serine-substituted version of that peptide was also detected is plotted for each sample. Two or three independent cultures were analyzed for each genotype and are plotted as separate bars. MS spectra were searched for the indicated modification (e.g. F to S). Data on absolute number of peptides identified in each sample are in Supplementary Table S4. (**B**) Relationship between toxicity and misincorporation. The fraction of substituted peptides detected is plotted against the growth rate of each strain relative to the control strain expressing tS* with the wild type anticodon. The vertical line indicates the background level of substitution detected in the control sample in the T to S search. Strains are colored as in A.

yielded a substantially higher rate of serine substitutions than the control sample expressing tS*(CGA), with the exception of the tS*-D(GUC) sample in the Ile to Ser search and to a lesser extent the tS*-I(GAU) sample in the Asp to Ser search (Supplementary Figure S5B). As Ile and Asp are <2 Da apart in mass and the correct substitution was not present in the search database, it is likely that true Asp to Ser substitutions were misidentified as Ile to Ser substitutions (and vice versa in the case of the tS*-I(GAU) sample). Supporting this hypothesis, 98% of the peptides from the tS*-D(GUC) sample that matched an Ile to Ser substitution were also found to have an Asp to Ser substitution in the correct Asp to Ser search. Similarly, 93% of the peptides from the tS*-I(GAU) sample that matched an Asp to Ser substitution were also found to have an Ile to Ser substitution in the correct search.

As a final control, we investigated whether the serine substitutions we observed occurred at the codons expected to be decoded by each tRNA. We compared the fraction of serine substitutions at each codon of the amino acid in question to the fraction of that codon in all peptides detected in the same MS dataset (to control for biases in codon use in highly expressed genes, which are more likely to be detected by MS) (Supplementary Figure S5C). tS*-F(GAA), tS*-C(GCA), and tS*-D(GUC) would be expected to decode both codons for their respective amino acid, and indeed we found serine substitutions at sites encoded by both codons. tS*-I(GAU) should decode only AUC and AUU codons and not AUA, and as expected nearly all serine substitutions were at sites encoded by AUC and AUU. tS*-P(CGG) should decode only CCG codons, and we found a strong enrichment for serine substitutions at CCG sites, which is the least used proline codon and is rare in the background set. Approximately 65–70% of Pro to Ser substitutions were at CCG, compared to 7% of all proline sites in our dataset. The 30% of Pro to Ser substitutions not at CCG may largely be false identifications, as the Pro to Ser search had a higher level of background than the other searches. However, they may also represent true instances of unexpected decoding.

The fraction of peptides with serine substitutions did not correlate well with the level of toxicity (Figure 3B). Most strikingly, yeast cells expressing tS*-C(GCA) had only a minor growth defect, but had the highest fraction of serine-substituted peptides detected. Although cysteine is one of the rarest amino acids in the proteome, we identified approximately the same absolute number of cysteine to serine substituted peptides in the tS*-C(GCA) strain as proline to serine in the more toxic tS*-P(CGG) strain (an average of 174 proline to serine substitutions and 211 cysteine to serine substitutions after filtering). Similarly, isoleucine and aspartic acid are equivalently abundant in the proteome and both tS*-D(GUC) and tS*-I(GAU) were extremely toxic, but cells expressing tS*-D(GUC) had nearly double the fraction of substituted peptides as those expressing tS*-I(GAU). These results show that amino acids can be replaced by serine to different extents when equivalently toxic, and even variants that are not toxic may have substantial levels of serine misincorporation. In summary, we conclude that toxic tRNA anticodon variants produce misincorporation, but that toxicity is not a direct measure of the degree of misincorporation.

Finally, we sought to generalize the method of conditional accumulation of a toxic tRNA to another tRNA species. The C5U mutation in the acceptor stem of a $tRNA^{Tyr}$ ochre suppressor provokes RTD, since this variant has reduced tRNA levels in a wild type strain relative to its levels in a *met22Δ* strain (48). We engineered the C5U mutation and a GCA anticodon into tY(GUA) to make the construct tY*-C(GCA) and integrated this construct into a *met22Δ* [*URA3 CEN MET22*] strain (Figure 4A). Although the tY*-C(GCA) variant was not toxic at 30°C when *MET22* was selected against with 5-FOA, it was

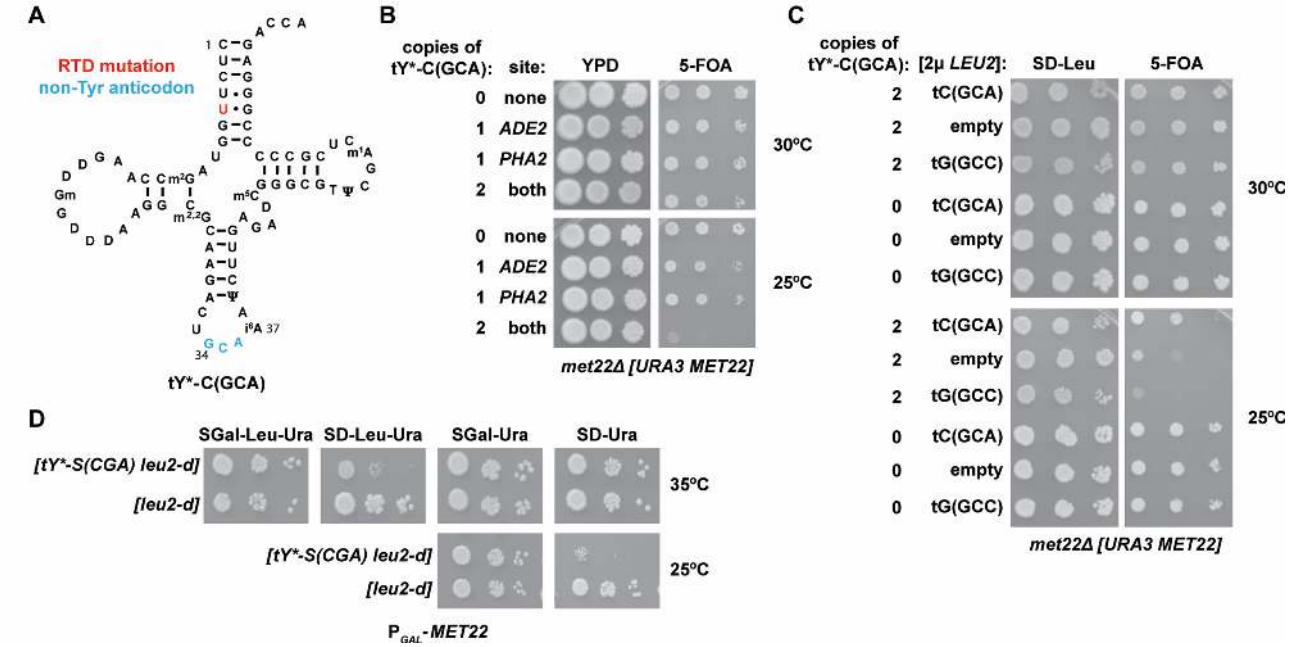

Figure 4. Generation of a conditionally toxic tRNA on a tY(GUA) scaffold. (**A**) Structure of the conditionally toxic tRNA tY*-C(GCA). (**B**) Expression of two copies of tY*-C(GCA) integrated at *ADE2* and *PHA2* causes toxicity in a *met22Δ* [CEN *URA3 MET22*] strain on media containing 5-FOA at 25°C. (**C**) Overexpression of wild type tRNA tC(GCA) rescues the toxicity of the yeast strain expressing two copies of tY*-C(GCA). (**D**) Overexpression of tY*-S(CGA) on a high copy [2μ *leu2-d*] plasmid causes toxicity at 25°C in a P_{GAL}-*MET22* strain on glucose-containing medium lacking uracil. When the copy number of the [2μ *leu2-d tY*-S(CGA)*] plasmid is further increased by growth in glucose-containing medium lacking uracil and leucine, it also causes toxicity at 35°C.

slightly toxic at 25°C; moreover, adding a second copy of the tY*-C(GCA) gene led to substantial toxicity at 25°C in the absence of Met22 (Figure 4B). Since the toxicity due to tY*-C(GCA) could be suppressed by overproduction of tC(GCA), but not tG(GCC) (Figure 4C), we infer that toxicity was due to insertion of tyrosine at cysteine codons. We also found that tY*-S(CGA) caused toxicity if it was overexpressed. Expression of tY*-S(CGA) on a high copy [2μ *URA3*$^+$ *leu2-d*] plasmid in a P_{GAL}-*MET22* strain resulted in toxicity on glucose-containing media lacking uracil at 25°C, when RTD is off. In glucose-containing media lacking both uracil and leucine, conditions in which RTD is off and the copy number of the [2μ *URA3*$^+$ *leu2-d*] plasmid is further increased several-fold (68), expression of tY*-S(CGA) was toxic at 35°C (Figure 4D). These results suggest that toxicity can be extended to multiple other tY* variants with different codons by increasing their expression, and further emphasize that this methodology of generating toxic tRNAs can be extended to any tRNA for which mutations sensitizing the tRNA to RTD can be identified.

DISCUSSION

In this work, we demonstrate that yeast strains can be made to conditionally accumulate toxic tRNAs via shutoff of the rapid tRNA decay pathway. By incorporating RTD-sensitizing mutations into the acceptor stem of $tRNA^{Ser(CGA)}$, we could analyze the effects of a comprehensive set of mutations in the anticodon on tRNA toxicity. Using both a library approach and individual assays, we showed that many of the anticodons lead to toxic tRNAs that would be lethal if they accumulated constitutively. We used these toxic tRNAs to investigate tRNA sequence–function relationships, and as a tool to conditionally allow proteome-wide misincorporation to study the effects of amino acid substitutions on fitness. We also showed that this methodology for conditional accumulation of toxic tRNAs was generalizable, since it could be applied to a $tRNA^{Tyr}$ variant that was subject to RTD, and since toxicity could be magnified by overexpression of the tRNA.

Our analysis showed large variations in the toxicity of anticodons in the tS* background. In the absence of Met22, 11 of the 64 possible anticodons resulted in highly toxic tRNAs, and another 18 caused a substantial growth disadvantage; such growth defects were largely ameliorated when Met22 was expressed to restore the RTD pathway. Moreover, although some anticodon sequences conferred strong toxicity, others did not even though they decode alternative codons for the same amino acid and in some cases have similar or higher codon usage.

Five factors in addition to codon usage likely contribute to the observed differences in toxicity among different variants. First, toxicity will vary depending on the number of competing wild type tRNAs, consistent with our demonstration that overproduction of cognate tRNAs but not control tRNAs reduced the toxicity of tS*-F(GAA), tS*-I(GUA), and tY*-C(GCA) variants. Second, toxicity is expected to vary depending on anticodon-codon pairing efficiency; for example, toxicity might be increased if the anticodon of the toxic tRNA pairs exactly with the codon in cases where the natural tRNA relies on weaker wobble pairing (69). Third, toxicity might be reduced for amino acids

whose tRNA synthetase predominantly recognizes the anticodon, and can compete with the seryl tRNA synthetase for charging when that anticodon is on the tS* scaffold. This competition could be a factor in explaining the lack of toxicity and lack of serine misincorporation in cells expressing tS*-T(AGU), for which the anticodon residues G35 and U36 are important identity determinants (70). A similar consideration might also explain why tY* variants were toxic only when overexpressed, as each of the anticodon residues of $tRNA^{Tyr}$ is a significant determinant in TyrRS recognition (71). Fourth, toxicity depends on the chemical differences between the mischarged amino acid and the amino acid normally specified by the codon. Thus, the lack of toxicity of tS*-C(GCA) might be due to the chemical and structural similarity between serine and cysteine, allowing serine to substitute for cysteine at many positions without untoward consequences. Fifth, anticodon loop modifications may modulate toxicity of anticodon variants, since modifications are prevalent in this loop and have a profound effect on decoding (72,73), and the required determinants for loop modification can depend on residues and features other than those in the anticodon itself (74,75). Although the explanation for why one anticodon is more toxic than another is likely due to a combination of factors, for any given anticodon this system permits the investigation of the cause of the toxicity.

It is not known why anticodon variants were more toxic if followed by A37 rather than G37, but substantial differences in toxicity were observed for four of ten variants examined with A37 and G37; the other six cases resulted in no difference or a mildly more toxic A37 variant. The four cases with substantially more A37 toxicity were anticodons for which G37 is the natural purine at residue 37. In these cases, G37 might be a determinant for cognate charging of tRNAs with that anticodon, which would reduce mischarging with serine and the consequent toxicity. Alternatively, A37 might act as a determinant for serine charging in addition to the variable loop, or G37 might be an antideterminant for charging by SerRS.

We also consistently observed that most variants were more toxic at lower temperatures. This increased toxicity is almost certainly due to the increased tRNA stability at lower temperatures, since we have observed RTD-dependent temperature sensitivity in mutants lacking specific modifications (43–47). Indeed, a large-scale study of temperature sensitivity of tRNA variants found that most temperature-sensitive variants are also substrates for the Met22-dependent tRNA decay pathway, suggesting that RTD is largely responsible for temperature-dependent tRNA decay (49).

One of the most surprising results of our experiments is that toxicity of a tS* variant anticodon was not a direct measure of the degree of misincorporation it produced. Although both tS*-T(AGU) and tS*-C(GCA) caused little toxicity, tS*-T(AGU) produced no measurable serine misincorporation at threonine codons, while tS*-C(GCA) resulted in high levels of serine misincorporation at cysteine codons. These results are consistent with the reported replacement of 20% of the valine residues in *E. coli* with the chemically similar α-aminobutyrate, albeit with growth defects (76), and a recent study in which 20 codons could be partially reassigned to tyrosine in *E. coli* with only modest growth defects (77). The lack of toxicity of serine misincorporation at cysteine codons also complicates the use of toxicity as a proxy for decoding efficiency, as both the inability to decode and the insensitivity of the cell to the resulting misincorporation can result in a lack of toxicity.

The lack of toxicity of amino acid swaps such as cysteine to serine has implications for the evolution of the genetic code and the tolerance of organisms to misincorporation. Examples have been identified of adaptive misincorporation, such as methionine misincorporation during oxidative stress in mammalian cells, misincorporation that improves protein activity at low temperatures in the thermophile *Aeropyrum pernix*, and increased misincorporation accelerating drug resistance in *Candida* and *E. coli* (78–82). In addition, a single deleterious leucine to proline mutation can be rescued by a suppressor tRNA that inserts alanine at proline codons, despite presumably also causing global misincorporation (83). Our finding that some types of misincorporation are well tolerated even at relatively high levels adds support to the hypothesis that misincorporation can be harnessed for beneficial functions. Although the genetic code is ancestral to all life, there are known alterations such as the translation of CUG as serine in *Candida* yeast and as alanine in *Pachysolen tannophilus* (84). A debate remains about how the genetic code is able to evolve given that any change would affect the whole proteome. One hypothesis requires an ambiguous intermediate in which a codon can be read simultaneously as multiple amino acids (85). This hypothesis has been supported by recent work showing that ambiguous tRNAs with additional attenuating mutations can produce mistranslation without a cost to fitness (86). Our results further suggest that for some amino acid substitutions, ambiguity can occur with minimal organismal toxicity, potentially allowing for a gradual shift in the meaning of a codon or flexibility in reading the genetic code.

These experiments provide a framework for four sets of future studies. First, this system can be used for studying the effects on the proteome of global replacement of each of several different amino acids with serine or tyrosine. Engineered misincorporation has been used in *E. coli*, yeast and mammalian cells to induce proteotoxic stress to identify cellular responses to protein misfolding and modifiers of protein quality control (31,87,88). This system will further facilitate studies into the cellular response to translational errors. Second, this system can be used for studying aspects of tRNA biology. Conditions in which misincorporation is toxic are useful for screens to identify modifiers of tRNA function. Indeed, we showed that a number of mutations in the serine tRNA itself can impair function and relieve toxicity, enabling a direct selection against functional regions of a toxic tRNA. In future experiments, this system could be used to investigate the effects of base modifications or natural tRNA variants on function for many different tRNA genes. High throughput mutagenesis has elucidated tRNA bases that are necessary for function, but the method used previously is limited to tRNAs that can be turned into nonsense suppressors or to single copy tRNA genes that are essential for normal growth (48,89). In contrast, our method is in principle generalizable to any tRNA that can be made conditionally toxic by making it a substrate for RTD, or

by conditional expression of other modulators of tRNA charging. Many tRNAs do not have absolute sequence requirements at the anticodon for charging, so it is likely that they will also be amenable to this method (6). Third, this method could be used to generate tRNAs that insert unnatural amino acids at sense codons for genetic code expansion, which may be useful in engineering tRNA species that cannot be turned into nonsense suppressors. Fourth, it is possible that this methodology could be employed to selectively kill cells, similarly to the toxic $tRNA^{Ser}$ species previously employed to kill tumor cells (90).

In summary, we present an approach to use conditionally toxic tRNAs as tools to induce misincorporation and probe the relationship between tRNA sequence and function. Our approach should be applicable to many problems in tRNA biology and to studying the effects of misincorporation on cellular physiology and evolution.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

ACKNOWLEDGEMENTS

We thank Elizabeth Grayhack and Lu Han for valuable discussions and comments during the course of this work. We thank Judit Villén and Ricard Rodriguez for help with the mass spectrometry experiments.

FUNDING

NIH [GM052347 to E.M.P. and GM103533 to S.F.]; W.M. Keck Foundation (to S.F.); S.F. is an investigator of the Howard Hughes Medical Institute; S.M.Z. is a Washington Research Foundation fellow of the Life Sciences Research Foundation; National Science Foundation Graduate Research Fellowship [DGE-1256082 to B.Y.R.]. Funding for open access charge: NIH [GM052347].
Conflict of interest statement. None declared.

REFERENCES

1. Sonenberg,N. and Hinnebusch,A.G. (2009) Regulation of translation initiation in eukaryotes: mechanisms and biological targets. *Cell*, **136**, 731–745.
2. Ling,J., Reynolds,N. and Ibba,M. (2009) Aminoacyl-tRNA synthesis and translational quality control. *Annu. Rev. Microbiol.*, **63**, 61–78.
3. Gromadski,K.B. and Rodnina,M.V. (2004) Kinetic determinants of high-fidelity tRNA discrimination on the ribosome. *Mol. Cell*, **13**, 191–200.
4. Gromadski,K.B., Daviter,T. and Rodnina,M.V. (2006) A uniform response to mismatches in codon–anticodon complexes ensures ribosomal fidelity. *Mol. Cell*, **21**, 369–377.
5. Wohlgemuth,I., Pohl,C., Mittelstaet,J., Konevega,A.L. and Rodnina,M.V. (2011) Evolutionary optimization of speed and accuracy of decoding on the ribosome. *Philos. Trans. R Soc. Lond. B Biol. Sci.*, **366**, 2979–2986.
6. Giege,R., Sissler,M. and Florentz,C. (1998) Universal rules and idiosyncratic features in tRNA identity. *Nucleic Acids Res.*, **26**, 5017–5035.
7. Putz,J., Florentz,C., Benseler,F. and Giege,R. (1994) A single methyl group prevents the mischarging of a tRNA. *Nat. Struct. Biol.*, **1**, 580–582.
8. Muramatsu,T., Nishikawa,K., Nemoto,F., Kuchino,Y., Nishimura,S., Miyazawa,T. and Yokoyama,S. (1988) Codon and amino-acid specificities of a transfer RNA are both converted by a single post-transcriptional modification. *Nature*, **336**, 179–181.
9. Rudinger,J., Florentz,C. and Giege,R. (1994) Histidylation by yeast HisRS of tRNA or tRNA-like structure relies on residues -1 and 73 but is dependent on the RNA context. *Nucleic Acids Res.*, **22**, 5031–5037.
10. Nameki,N., Asahara,H., Shimizu,M., Okada,N. and Himeno,H. (1995) Identity elements of Saccharomyces cerevisiae tRNA(His). *Nucleic Acids Res.*, **23**, 389–394.
11. Boniecki,M.T., Vu,M.T., Betha,A.K. and Martinis,S.A. (2008) CP1-dependent partitioning of pretransfer and posttransfer editing in leucyl-tRNA synthetase. *Proc. Natl. Acad. Sci. U.S.A.*, **105**, 19223–19228.
12. Sarkar,J. and Martinis,S.A. (2011) Amino-acid-dependent shift in tRNA synthetase editing mechanisms. *J. Am. Chem. Soc.*, **133**, 18510–18513.
13. Jakubowski,H. (2012) Quality control in tRNA charging. *Wiley Interdiscipl. Rev. RNA*, **3**, 295–310.
14. Ahel,I., Korencic,D., Ibba,M. and Soll,D. (2003) Trans-editing of mischarged tRNAs. *Proc. Natl. Acad. Sci. U.S.A.*, **100**, 15422–15427.
15. Murphy,F.V.T. and Ramakrishnan,V. (2004) Structure of a purine-purine wobble base pair in the decoding center of the ribosome. *Nat. Struct. Mol. Biol.*, **11**, 1251–1252.
16. Kothe,U. and Rodnina,M.V. (2007) Codon reading by tRNAAla with modified uridine in the wobble position. *Mol. Cell*, **25**, 167–174.
17. Demeshkina,N., Jenner,L., Westhof,E., Yusupov,M. and Yusupova,G. (2012) A new understanding of the decoding principle on the ribosome. *Nature*, **484**, 256–259.
18. Rozov,A., Demeshkina,N., Khusainov,I., Westhof,E., Yusupov,M. and Yusupova,G. (2016) Novel base-pairing interactions at the tRNA wobble position crucial for accurate reading of the genetic code. *Nat. Commun.*, **7**, 10457.
19. Rozov,A., Demeshkina,N., Westhof,E., Yusupov,M. and Yusupova,G. (2015) Structural insights into the translational infidelity mechanism. *Nat. Commun.*, **6**, 7251.
20. Cochella,L. and Green,R. (2005) An active role for tRNA in decoding beyond codon:anticodon pairing. *Science*, **308**, 1178–1180.
21. Liu,Y., Satz,J.S., Vo,M.N., Nangle,L.A., Schimmel,P. and Ackerman,S.L. (2014) Deficiencies in tRNA synthetase editing activity cause cardioproteinopathy. *Proc. Natl. Acad. Sci. U.S.A.*, **111**, 17570–17575.
22. Lee,J.W., Beebe,K., Nangle,L.A., Jang,J., Longo-Guess,C.M., Cook,S.A., Davisson,M.T., Sundberg,J.P., Schimmel,P. and Ackerman,S.L. (2006) Editing-defective tRNA synthetase causes protein misfolding and neurodegeneration. *Nature*, **443**, 50–55.
23. Antonellis,A., Ellsworth,R.E., Sambuughin,N., Puls,I., Abel,A., Lee-Lin,S.Q., Jordanova,A., Kremensky,I., Christodoulou,K., Middleton,L.T. *et al.* (2003) Glycyl tRNA synthetase mutations in Charcot-Marie-Tooth disease type 2D and distal spinal muscular atrophy type V. *Am. J. Hum. Genet.*, **72**, 1293–1299.
24. Meyer-Schuman,R. and Antonellis,A. (2017) Emerging mechanisms of aminoacyl-tRNA synthetase mutations in recessive and dominant human disease. *Hum. Mol. Genet.*, **26**, R114–R127.
25. Stansfield,I., Jones,K.M., Herbert,P., Lewendon,A., Shaw,W.V. and Tuite,M.F. (1998) Missense translation errors in Saccharomyces cerevisiae. *J. Mol. Biol.*, **282**, 13–24.
26. Kramer,E.B. and Farabaugh,P.J. (2007) The frequency of translational misreading errors in E. coli is largely determined by tRNA competition. *RNA*, **13**, 87–96.
27. Kramer,E.B., Vallabhaneni,H., Mayer,L.M. and Farabaugh,P.J. (2010) A comprehensive analysis of translational missense errors in the yeast Saccharomyces cerevisiae. *RNA*, **16**, 1797–1808.
28. Drummond,D.A. and Wilke,C.O. (2009) The evolutionary consequences of erroneous protein synthesis. *Nat. Rev. Genet.*, **10**, 715–724.
29. Nangle,L.A., Motta,C.M. and Schimmel,P. (2006) Global effects of mistranslation from an editing defect in mammalian cells. *Chem. Biol.*, **13**, 1091–1100.
30. Santos,M., Pereira,P.M., Varanda,A.S., Carvalho,J., Azevedo,M., Mateus,D.D., Mendes,N., Oliveira,P., Trindade,F., Pinto,M.T. *et al.* (2018) Codon misreading tRNAs promote tumor growth in mice. *RNA Biol.*, 1–14.
31. Ruan,B., Palioura,S., Sabina,J., Marvin-Guy,L., Kochhar,S., Larossa,R.A. and Soll,D. (2008) Quality control despite mistranslation caused by an ambiguous genetic code. *Proc. Natl. Acad. Sci. U.S.A.*, **105**, 16502–16507.

32. Santos,M.A., Keith,G. and Tuite,M.F. (1993) Non-standard translational events in Candida albicans mediated by an unusual seryl-tRNA with a 5′-CAG-3′ (leucine) anticodon. *EMBO J.*, **12**, 607–616.
33. Santos,M.A. and Tuite,M.F. (1995) The CUG codon is decoded in vivo as serine and not leucine in Candida albicans. *Nucleic Acids Res.*, **23**, 1481–1486.
34. Massey,S.E., Moura,G., Beltrao,P., Almeida,R., Garey,J.R., Tuite,M.F. and Santos,M.A. (2003) Comparative evolutionary genomics unveils the molecular mechanism of reassignment of the CTG codon in Candida spp. *Genome Res.*, **13**, 544–557.
35. Gomes,A.C., Miranda,I., Silva,R.M., Moura,G.R., Thomas,B., Akoulitchev,A. and Santos,M.A. (2007) A genetic code alteration generates a proteome of high diversity in the human pathogen Candida albicans. *Genome Biol.*, **8**, R206.
36. Javid,B., Sorrentino,F., Toosky,M., Zheng,W., Pinkham,J.T., Jain,N., Pan,M., Deighan,P. and Rubin,E.J. (2014) Mycobacterial mistranslation is necessary and sufficient for rifampicin phenotypic resistance. *Proc. Natl. Acad. Sci. U.S.A.*, **111**, 1132–1137.
37. Li,L., Boniecki,M.T., Jaffe,J.D., Imai,B.S., Yau,P.M., Luthey-Schulten,Z.A. and Martinis,S.A. (2011) Naturally occurring aminoacyl-tRNA synthetases editing-domain mutations that cause mistranslation in Mycoplasma parasites. *Proc. Natl. Acad. Sci. U.S.A.*, **108**, 9378–9383.
38. Meyerovich,M., Mamou,G. and Ben-Yehuda,S. (2010) Visualizing high error levels during gene expression in living bacterial cells. *Proc. Natl. Acad. Sci. U.S.A.*, **107**, 11543–11548.
39. Young,D.D. and Schultz,P.G. (2018) Playing with the molecules of life. *ACS Chem. Biol.*, **13**, 854–870.
40. Schramm,L. and Hernandez,N. (2002) Recruitment of RNA polymerase III to its target promoters. *Genes Dev.*, **16**, 2593–2620.
41. Geiduschek,E.P. and Tocchini-Valentini,G.P. (1988) Transcription by RNA polymerase III. *Annu. Rev. Biochem.*, **57**, 873–914.
42. Marck,C., Kachouri-Lafond,R., Lafontaine,I., Westhof,E., Dujon,B. and Grosjean,H. (2006) The RNA polymerase III-dependent family of genes in hemiascomycetes: comparative RNomics, decoding strategies, transcription and evolutionary implications. *Nucleic Acids Res.*, **34**, 1816–1835.
43. Alexandrov,A., Chernyakov,I., Gu,W., Hiley,S.L., Hughes,T.R., Grayhack,E.J. and Phizicky,E.M. (2006) Rapid tRNA decay can result from lack of nonessential modifications. *Mol. Cell*, **21**, 87–96.
44. Kotelawala,L., Grayhack,E.J. and Phizicky,E.M. (2008) Identification of yeast tRNA Um(44) 2′-O-methyltransferase (Trm44) and demonstration of a Trm44 role in sustaining levels of specific tRNA(Ser) species. *RNA*, **14**, 158–169.
45. Chernyakov,I., Whipple,J.M., Kotelawala,L., Grayhack,E.J. and Phizicky,E.M. (2008) Degradation of several hypomodified mature tRNA species in Saccharomyces cerevisiae is mediated by Met22 and the 5′-3′ exonucleases Rat1 and Xrn1. *Genes Dev.*, **22**, 1369–1380.
46. Dewe,J.M., Whipple,J.M., Chernyakov,I., Jaramillo,L.N. and Phizicky,E.M. (2012) The yeast rapid tRNA decay pathway competes with elongation factor 1A for substrate tRNAs and acts on tRNAs lacking one or more of several modifications. *RNA*, **18**, 1886–1896.
47. Whipple,J.M., Lane,E.A., Chernyakov,I., D'Silva,S. and Phizicky,E.M. (2011) The yeast rapid tRNA decay pathway primarily monitors the structural integrity of the acceptor and T-stems of mature tRNA. *Genes Dev.*, **25**, 1173–1184.
48. Guy,M.P., Young,D.L., Payea,M.J., Zhang,X., Kon,Y., Dean,K.M., Grayhack,E.J., Mathews,D.H., Fields,S. and Phizicky,E.M. (2014) Identification of the determinants of tRNA function and susceptibility to rapid tRNA decay by high-throughput in vivo analysis. *Genes Dev.*, **28**, 1721–1732.
49. Payea,M.J., Sloma,M.F., Kon,Y., Young,D.L., Guy,M.P., Zhang,X., De Zoysa,T., Fields,S., Mathews,D.H. and Phizicky,E.M. (2018) Widespread temperature sensitivity and tRNA decay due to mutations in a yeast tRNA. *RNA*, **24**, 410–422.
50. Janke,C., Magiera,M.M., Rathfelder,N., Taxis,C., Reber,S., Maekawa,H., Moreno-Borchart,A., Doenges,G., Schwob,E., Schiebel,E. *et al.* (2004) A versatile toolbox for PCR-based tagging of yeast genes: new fluorescent proteins, more markers and promoter substitution cassettes. *Yeast*, **21**, 947–962.
51. Gietz,R.D. and Schiestl,R.H. (2007) High-efficiency yeast transformation using the LiAc/SS carrier DNA/PEG method. *Nat. Protoc.*, **2**, 31–34.
52. Langmead,B. and Salzberg,S.L. (2012) Fast gapped-read alignment with Bowtie 2. *Nat. Methods*, **9**, 357–359.
53. Ghaemmaghami,S., Huh,W.K., Bower,K., Howson,R.W., Belle,A., Dephoure,N., O'Shea,E.K. and Weissman,J.S. (2003) Global analysis of protein expression in yeast. *Nature*, **425**, 737–741.
54. Johansson,M.J., Esberg,A., Huang,B., Bjork,G.R. and Bystrom,A.S. (2008) Eukaryotic wobble uridine modifications promote a functionally redundant decoding system. *Mol. Cell. Biol.*, **28**, 3301–3312.
55. Rappsilber,J., Mann,M. and Ishihama,Y. (2007) Protocol for micro-purification, enrichment, pre-fractionation and storage of peptides for proteomics using StageTips. *Nat. Protoc.*, **2**, 1896–1906.
56. Eng,J.K., Jahan,T.A. and Hoopmann,M.R. (2013) Comet: an open-source MS/MS sequence database search tool. *Proteomics*, **13**, 22–24.
57. Elias,J.E. and Gygi,S.P. (2007) Target-decoy search strategy for increased confidence in large-scale protein identifications by mass spectrometry. *Nat. Methods*, **4**, 207–214.
58. Kall,L., Canterbury,J.D., Weston,J., Noble,W.S. and MacCoss,M.J. (2007) Semi-supervised learning for peptide identification from shotgun proteomics datasets. *Nat. Methods*, **4**, 923–925.
59. Eng,J.K., Hoopmann,M.R., Jahan,T.A., Egertson,J.D., Noble,W.S. and MacCoss,M.J. (2015) A deeper look into Comet–implementation and features. *J. Am. Soc. Mass Spectrom.*, **26**, 1865–1874.
60. Himeno,H., Yoshida,S., Soma,A. and Nishikawa,K. (1997) Only one nucleotide insertion to the long variable arm confers an efficient serine acceptor activity upon Saccharomyces cerevisiae tRNA(Leu) in vitro. *J. Mol. Biol.*, **268**, 704–711.
61. Jansen,R., Bussemaker,H.J. and Gerstein,M. (2003) Revisiting the codon adaptation index from a whole-genome perspective: analyzing the relationship between gene expression and codon occurrence in yeast using a variety of models. *Nucleic Acids Res.*, **31**, 2242–2251.
62. Henikoff,S. and Henikoff,J.G. (1992) Amino acid substitution matrices from protein blocks. *Proc. Natl. Acad. Sci. U.S.A.*, **89**, 10915–10919.
63. Konevega,A.L., Soboleva,N.G., Makhno,V.I., Semenkov,Y.P., Wintermeyer,W., Rodnina,M.V. and Katunin,V.I. (2004) Purine bases at position 37 of tRNA stabilize codon–anticodon interaction in the ribosomal A site by stacking and Mg2+-dependent interactions. *RNA*, **10**, 90–101.
64. Chan,P.P. and Lowe,T.M. (2016) GtRNAdb 2.0: an expanded database of transfer RNA genes identified in complete and draft genomes. *Nucleic Acids Res.*, **44**, D184–D189.
65. Santos,M.A., Perreau,V.M. and Tuite,M.F. (1996) Transfer RNA structural change is a key element in the reassignment of the CUG codon in Candida albicans. *EMBO J.*, **15**, 5060–5068.
66. Marck,C. and Grosjean,H. (2002) tRNomics: analysis of tRNA genes from 50 genomes of Eukarya, Archaea, and Bacteria reveals anticodon-sparing strategies and domain-specific features. *RNA*, **8**, 1189–1232.
67. Gautheret,D., Damberger,S.H. and Gutell,R.R. (1995) Identification of base-triples in RNA using comparative sequence analysis. *J. Mol. Biol.*, **248**, 27–43.
68. Erhart,E. and Hollenberg,C.P. (1983) The presence of a defective LEU2 gene on 2 mu DNA recombinant plasmids of Saccharomyces cerevisiae is responsible for curing and high copy number. *J. Bacteriol.*, **156**, 625–635.
69. Letzring,D.P., Dean,K.M. and Grayhack,E.J. (2010) Control of translation efficiency in yeast by codon–anticodon interactions. *RNA*, **16**, 2516–2528.
70. Nameki,N. (1995) Identity elements of tRNA(Thr) towards Saccharomyces cerevisiae threonyl-tRNA synthetase. *Nucleic Acids Res.*, **23**, 2831–2836.
71. Fechter,P., Rudinger-Thirion,J., Theobald-Dietrich,A. and Giege,R. (2000) Identity of tRNA for yeast tyrosyl-tRNA synthetase: tyrosylation is more sensitive to identity nucleotides than to structural features. *Biochemistry*, **39**, 1725–1733.
72. Murphy,F.V.T., Ramakrishnan,V., Malkiewicz,A. and Agris,P.F. (2004) The role of modifications in codon discrimination by tRNA(Lys)UUU. *Nat. Struct. Mol. Biol.*, **11**, 1186–1191.
73. Agris,P.F., Vendeix,F.A. and Graham,W.D. (2007) tRNA's wobble decoding of the genome: 40 years of modification. *J. Mol. Biol.*, **366**, 1–13.

74. Han,L., Marcus,E., D'Silva,S. and Phizicky,E.M. (2017) S. cerevisiae Trm140 has two recognition modes for 3-methylcytidine modification of the anticodon loop of tRNA substrates. *RNA*, **23**, 406–419.
75. Arimbasseri,A.G., Iben,J., Wei,F.Y., Rijal,K., Tomizawa,K., Hafner,M. and Maraia,R.J. (2016) Evolving specificity of tRNA 3-methyl-cytidine-32 (m3C32) modification: a subset of tRNAsSer requires N6-isopentenylation of A37. *RNA*, **22**, 1400–1410.
76. Doring,V., Mootz,H.D., Nangle,L.A., Hendrickson,T.L., de Crecy-Lagard,V., Schimmel,P. and Marliere,P. (2001) Enlarging the amino acid set of Escherichia coli by infiltration of the valine coding pathway. *Science*, **292**, 501–504.
77. Schmitt,M.A., Biddle,W. and Fisk,J.D. (2018) Mapping the plasticity of the escherichia coli genetic code with orthogonal Pair-Directed sense codon reassignment. *Biochemistry*, **57**, 2762–2774.
78. Netzer,N., Goodenbour,J.M., David,A., Dittmar,K.A., Jones,R.B., Schneider,J.R., Boone,D., Eves,E.M., Rosner,M.R., Gibbs,J.S. *et al.* (2009) Innate immune and chemically triggered oxidative stress modifies translational fidelity. *Nature*, **462**, 522–526.
79. Bezerra,A.R., Simoes,J., Lee,W., Rung,J., Weil,T., Gut,I.G., Gut,M., Bayes,M., Rizzetto,L., Cavalieri,D. *et al.* (2013) Reversion of a fungal genetic code alteration links proteome instability with genomic and phenotypic diversification. *Proc. Natl. Acad. Sci. U.S.A.*, **110**, 11079–11084.
80. Weil,T., Santamaria,R., Lee,W., Rung,J., Tocci,N., Abbey,D., Bezerra,A.R., Carreto,L., Moura,G.R., Bayes,M. *et al.* (2017) Adaptive mistranslation accelerates the evolution of fluconazole resistance and induces major genomic and gene expression alterations in candida albicans. *mSphere*, **2**, e00167-17.
81. Bratulic,S., Toll-Riera,M. and Wagner,A. (2017) Mistranslation can enhance fitness through purging of deleterious mutations. *Nat. Commun.*, **8**, 15410.
82. Schwartz,M.H. and Pan,T. (2016) Temperature dependent mistranslation in a hyperthermophile adapts proteins to lower temperatures. *Nucleic Acids Res.*, **44**, 294–303.
83. Hoffman,K.S., Berg,M.D., Shilton,B.H., Brandl,C.J. and O'Donoghue,P. (2017) Genetic selection for mistranslation rescues a defective co-chaperone in yeast. *Nucleic Acids Res.*, **45**, 3407–3421.
84. Muhlhausen,S., Findeisen,P., Plessmann,U., Urlaub,H. and Kollmar,M. (2016) A novel nuclear genetic code alteration in yeasts and the evolution of codon reassignment in eukaryotes. *Genome Res.*, **26**, 945–955.
85. Schultz,D.W. and Yarus,M. (1994) Transfer RNA mutation and the malleability of the genetic code. *J. Mol. Biol.*, **235**, 1377–1380.
86. Berg,M.D., Hoffman,K.S., Genereaux,J., Mian,S., Trussler,R.S., Haniford,D.B., O'Donoghue,P. and Brandl,C.J. (2017) Evolving mistranslating tRNAs through a phenotypically ambivalent intermediate in saccharomyces cerevisiae. *Genetics*, **206**, 1865–1879.
87. Kalapis,D., Bezerra,A.R., Farkas,Z., Horvath,P., Bodi,Z., Daraba,A., Szamecz,B., Gut,I., Bayes,M., Santos,M.A. *et al.* (2015) Evolution of robustness to protein mistranslation by accelerated protein turnover. *PLoS Biol.*, **13**, e1002291.
88. Geslain,R., Cubells,L., Bori-Sanz,T., Alvarez-Medina,R., Rossell,D., Marti,E. and Ribas de Pouplana,L. (2010) Chimeric tRNAs as tools to induce proteome damage and identify components of stress responses. *Nucleic Acids Res.*, **38**, e30.
89. Li,C., Qian,W., Maclean,C.J. and Zhang,J. (2016) The fitness landscape of a tRNA gene. *Science*, **352**, 837–840.
90. Zhou,D.H., Lee,J., Frankenberger,C., Geslain,R., Rosner,M. and Pan,T. (2012) Anti-tumor effects of an engineered 'killer' transfer RNA. *Biochem. Biophys. Res. Commun.*, **427**, 148–153.